Enterobacterial responses to concerted nitrosative and oxidative stresses

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A thesis submitted for the degree of:

Doctor of Philosophy

January 2017

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The University of Sheffield

Abstract

Compounds that exert nitrosative and oxidative stress play key roles in the innate immune response and microbicidal activity. Survival and recovery after exposure to stresses encountered in the macrophage from nitric oxide, partially reduced oxygen species, hypochlorous acid and other chemicals may lead to infection and pathogenesis. This project aims to mimic some aspects of the environment encountered by pathogenic bacteria inside macrophages using *E. coli* as a model and study the response of stress-related genes after adding these stresses individually and/or simultaneously. Experiments were done to determine the effect of combinations of these three reagents on the growth and viability of *E coli* MG1655 and *hmp* mutant strains. The results showed that combined stresses caused a significant impact on the growth and viability of both strains. This study investigated the ability of *E coli* MG1655 wild-type and a mutant defective in the flavohaemoglobin (*hmp*) to recover from these stresses. The results indicated that both strains recovered when the three stresses were removed and the cells were transferred to fresh medium. RT-PCR data confirmed that DETA NONOate causes significant up-regulation of *hmp* (encoding the NO-detoxifying flavohaemoglobin) and *norV* (encoding an NO reductase), while H_2O_2 causes up-regulation of *katG* (catalase), *ahpC* (alkylhydroperoxide) and *sodA* (manganese superoxide dismutase). Expression of *nemA* was previously implicated in HOCl responses, and was also affected by this reagent. In addition, RT-PCR showed differential expression of genes in response to oxidative and nitrosative stresses when they were added simultaneously, compared to when they were added individually. Cells were treated with DETA NONOate (slow NO releaser) alone or NOC-5 plus NOC-7 (fast NO releasers) showed an increase in the expression of all tested oxidative and nitrosative stress-related genes, dependent on treatment time. Surprisingly, the *nemA* gene was also up-regulated in response to NO donors. Oxidative stress-related genes were highly up-regulated when cells were treated to combined stresses for 10 or 25 min relative to the individual stresses. On the other hand, treating cells with NO first, followed with H_2O_2

impaired the expression of *norV*. The *hmp* was highly up-regulated when cells were treated with combined stresses. Checkerboard studies were used to determine fractional inhibitory concentrations and revealed that NO in combination with cefotaxime, gentamicin and polymyxin B showed additive effects against multidrug-resistant uropathogenic *E. coli* (EC958). Moreover, the combination of NO and doxycycline showed antagonistic effects against *E.coli* MG1655, *hmp* mutant and EC958 strains. This study has contributed to understanding interaction between antibiotics and other antimicrobial agent when used simultaneously or sequentially. Since bacterial assault by multiple stress reagents is a hallmark of the macrophage environment, this work may contribute to an understanding of bacterial survival mechanism in infection and disease.

Acknowledgments

First and foremost, I would like to thank my supervisor Professor Robert Poole for his generous support and guidance throughout the course of my PhD. His continuous guidance helped me in the time of research and writing of this thesis.

I want to thank Dr. Helen Jesse for her support, help and encouragement during my PhD. Also many thanks to Hannah Southam for stimulating discussion and for on going advice. Thanks to Namrata Rana for her support all the time. To Thomas Smith for his help. I would like also to thank Dr. Mariana Tinajero-Trejo for her help and for enlightening me the first glance of research.

Thanks also to the Pooligans, past and present for their help, discussion and for sharing so many memorable moments outside the lab.

A special thank to my Mom and Dad for their constant love and help. Thank you for always supporting me and being always with me at every step. Also, many thanks to my sister and brothers for supporting me all the time.

To my beloved sons Ahmed and Faisal, Thank you for being patience, happy and good boys all the time. I love you.

Most of all I would like to thank my husband Mazen Sindi for his support, help and love throughout the four years of my PhD. I couldn't have done it without you.

Abbreviation

[Fe-S]	Iron-sulfur cluster
AhpCF	Peroxiredoxin alkyl hydroperoxide reductase
bNOS	Bacterial nitric oxide synthases
CNS	Central nervous system
CR3	Complement receptors
DETA NONOate	(Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate, 3,3-Bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene, 2,2'-(Hydroxynitrosohydrazino)bis-ethanamine
dH_2O	Distilled water
eNOS	Endothelial nitric oxide synthase
ESBL	Extended–spectrum β lactamase
FICs	Fractional inhibitory concentrations
FIRd	Flavorubredoxin
FMN	Flavinmononucleotide
GSNO	S-nitrosoglutathione
Hmp	Flavohaemoglobin
iNOS	Inducible nitric oxide synthase
KM	Kanamycin
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentrations
MPO	Myeloperoxidase
NEM	N-ethylmaleimide
NemA	N-ethylmaleimide reductase
NemR	N-ethylmaleimide reductase repressor

NK cells	Natural killer cells
NO^-	Nitroxyl anion
NO^+	Nitrosonium cation
NOC-5	1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene
NOC-7	1-Hydroxy-2-oxo-3-(N-3-methyl-aminopropyl)-3-methyl-1-triazene
NOC-18	DETA NONOate
NorV	Flavorubredoxin
NOHA	N^{ω}-hydroxy-L-arginine
NOS	Nitric oxide synthase
NrfA	Cytochrome *c* nitrite reductase
$ONOO^-$	Peroxinitrite
$ONOOCO_2^-$	Nitroperoxycarbonate
PAMPs	Pathogen-associated molecular patterns
Phox	Phagocyte oxidase
PMNLs	Polymorphonuclear lymphocytes
PRRs	Pattern recognition receptors
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TLR	Toll-like receptors
UPEC	Uropathogenic *E. coli*

Table of contents

Table of figures

List of tables

Chapter 1

Introduction

Chapter 1. Introduction

1.1 The immune response

Bacteria that gain entry to higher animals face numerous challenges to survival, growth and reproduction. For example, mammalian organisms produce iron-chelating components that reduce the availability of iron an element essential for all bacteria. In the gastrointestinal tract, high population of indigenous bacteria provides competition for nutrients, produce noxious metabolic by-products, and deplete oxygen to undetectable levels. Pathogenic bacteria face an array of host immune defenses.

1.1.1 Innate immunity

Innate immune response is a system which has developed through evolutionary processes as a protective force against infective agents when they first enter the body. The system is based around germline-encoded receptor groups as well as molecules capable of identifying conserved molecular patterns which are only present in microorganisms (Malhotra et al., 2000).

This innate immune system comprises a range of protective chemical, mechanical and cellular elements. The chemical components of the system have three subcomponents: First, the recognition molecule, which can be soluble molecule or cell associated. Secondly, chemokines and cytokines are responsible for organising immune-protective responses. Finally, peptides and proteins involve in bacterial elimination. The mechanical elements of the innate immune system are when pathogens are challenged physically by the mucosa and epidermis, which physically block infection, along with the movement of cilia, mucus secretions desquamation and motility. Cellular elements are the third elements of the innate immune system and this system includes the mast cells, epithelial cells, dendritic cells, Natural killer cells (NK cells), T cells, and phagocytic cells (granulocytes and macrophage cells) (Basset et al., 2003).

Pathogens are continually challenged when in a host's tissues by NK and phagocyticells, proteins of the blood, such as those which mediate inflammatory processes and complement-system proteins, and by cytokine proteins with

responsibility for regulation of the cells of the innate immune system (Iwasaki and Medzhitov, 2004).

Pathogenic agents can be identified by pattern recognition receptors or PRRs, due to the typically arranged pathogen-associated molecular patterns (PAMP) which they display. Such PAMPs represent fixed and essential constructions within microbial organisms which have not evolved quickly over time. Included in this are lipopolysaccharide (LPS), peptidoglycan, lipoprotein, oligosaccharides and lipoarabinomannan. Cellular immune system components such as epithelial cells, granulocytes, macrophage-monocytes, granulocytes, dendritic and mast cells each contain PRRs, which themselves are divided into groups, including scavenger receptors, formyl peptid receptors, CD14, Toll-like receptors (TLR), glycan and mannose receptors and complement receptors (CR3). From a range of receptors which are soluble and PAMP-binding, CD14 for example exists also as a cell-based PRR (Basset et al., 2003).

Natural killer cells develop from T cell receptors in the lymphoid line of cells and both granzyme and perforin are contained within them. These molecules can be lethal to tumour cells or cells with a viral infection when the NK cells come into direct contact with such cells (Middleton et al., 2002).

Phagocytic cells have a central role as effectors in the innate immune system, and act to destroy invading pathogens as they enter the host, to prevent them from spreading, as well as stimulating adaptive immunity by macrophage development of antigens. Localised macrophage cells respond to a threat initially, and where required will then recruit blood-based neutrophils (Zhang et al., 2000).

1.1.2 Macrophages and Neutrophils

The innate system of immune response in humans encompasses a range of cellular components, including neutrophils, mast cells, monocytes and macrophages, dendritic cells and natural killer cells, which secrete a range of mediators which are soluble. In combination, this mediator-cell system can effectively combat invading pathogens (Kumar and Sharma, 2010).

In mammals, parasitic, fungal and bacterial pathogens are identified and eliminated

via a complex system of immune defence. Phagocytosis is the principle mediator of this defence, allowing neutrophils and macrophages to envelop pathogens and use digestion via enzymes, as well as reactive oxygen species (ROS) and reactive nitrogen species (RNS), to destroy them (Fig 1.1) (reviewed in (Vatansever et al., 2013)). The innate immune cells found in mammals work in reaction to the presence of pathogens via activation of particular proteins, which then exert nitrosative and oxidative stress (Baptista et al., 2012).

The initial response by the innate immune system to pathogen invasion comes from the phagocytes. The antimicrobial action of phagocytes comes primarily from the system for NADPH phagocyte oxidase (phox), which allows the superoxide ($O_2^{\bullet-}$) to be produced, and from inducible nitric oxide synthase (iNOS) pathways, which synthesises nitric oxide ($NO^{\bullet}$) radicals (Fang, 2004). The two systems can act in tandem, and both rely upon molecular oxygen and NADPH. However, NADPH phagocyte oxidase and iNOS are different enzyme complexes and regulated separately. Further intermediates derived from oxygen which are capable of altering organic molecules are termed reactive oxygen species or ROS. Meanwhile, reactive nitrogen species or RNS include NO• as well as derivatives of this (Fang, 2004). Microbial pathogens are phagocytized by macrophages, which have a range of means for killing these microbes, such as by producing lysosomal enzymes, antimicrobial peptides, reactive oxygen species and reactive nitrogen species (Fig 1.1)(Fang, 2004). Nitric oxide (NO) is created by macrophages based upon a range of stimulating factors. Macrophages go on to alter NO and create NO_2, N_2O_3 and S-nitrosothiols (Fang, 1997), and reactions also occur with oxygen species, such as with $O_2^{\bullet-}$ in forming peroxynitrite ($ONOO^-$) (Ischiropoulos et al., 1992, Pryor and Squadrito, 1995) (Fig 1.1). These processes can all support the macrophage's ability to kill bacteria(Ischiropoulos et al., 1992). Neutrophils (polymorphonuclear lymphocytes or PMNLs) form a central part of innate immunity and set the resistance level of the host to a range of pathogenic bacteria and fungi. To strengthen their power to combat pathogens, a range of cytotoxic granules which contain varied molecules which act against microbes are present in these cells. These molecules include cationic peptides, myeloperoxidase, proteases and lactoferrin. Macrophage and neutrophil cells develop from a common source and there is functional overlap between the cells, with both using phagocytosis against invaders (Kumar and Sharma, 2010).

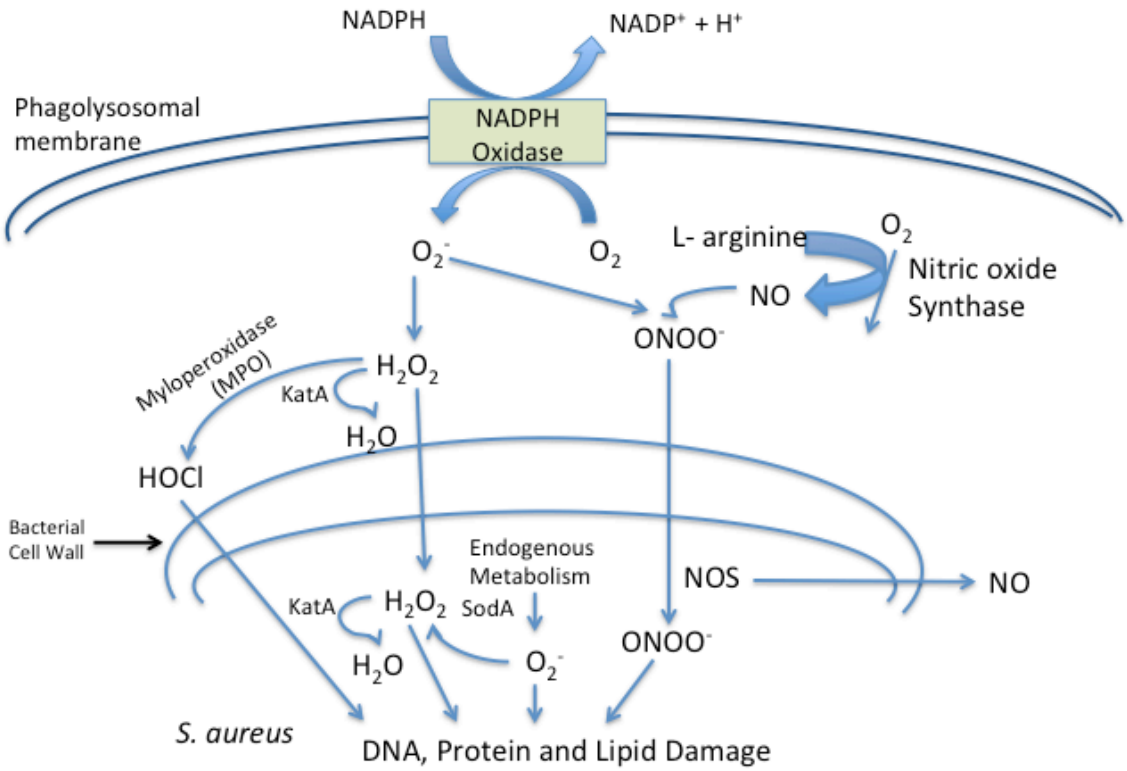


Figure 1.1 Neutrophils and macrophages generate ROS to defend against pathogenic microbes. Phagosomes envelop foreign organisms, stimulating NADPH oxidase to produce $O_2^{\bullet-}$, myeloperoxidase to produce HOCl, while iNOS produces NO. RNS and ROS jointly target microbes. Adapted from (Vatansever et al., 2013).

Expression of both iNOS and NADPH phagocyte oxidase occurs within mononuclear and polymorphonuclear phagocytes, while the ROS produced is greater in neutrophils than in macrophages, and macrophages generally have far greater RNS production (Nathan and Shiloh, 2000).

1.1.2.1 The nitrosative burst of phagocytic cells

The oxidative burst is followed by production of a nitrosative burst which occurs due to the actions of mediators of inflammation, as well as pathogenic agonists (Lowenstein et al., 1993). Different types of cells produce NO by the oxidation of arginine in aerobic conditions with specific cofactors. Nitric oxide synthase (NOS) catalyses this reaction (Stuehr, 1999). NOS can either enzymatically produce NO, or NO can arise through respiratory nitrite reductase (Clarke et al*.,* 2008). This is subject to mediation from the NO produced when nitric oxide synthase (iNOS) is activated. The two isoforms are expressed constitutively and depend upon calcium; once infection occurs, iNOS gives significantly greater amounts of NO in comparison to constitutive isoforms following infection (Lowenstein and Padalko, 2004). When considering microbes, iNOS is of greatest significance, and has been found across a range of different cells capable of stimulation, including neutrophils, macrophages, vascular smooth cells and glial cells within the central nervous system (CNS) (Bogdan, 2001). Where microbial infection is present, various actions are observed from the NO present. NO contributes to enhancement of host defence through antimicrobial and anti-inflammatory activity, as well as being involved in cytotoxic and proinflammatory functions (Fang, 2004). Failure of leucocyte phagocytes to synthesise NO has been linked to greater danger of infection from *Porphyromonas gingivalis* (Gyurko et al., 2003) *Trypanosoma cruzi* (Talvani et al., 2002).

NOSs, which cross multiple domains and are strongly regulated, were initially discovered within mammalian organisms. These synthases can be divided into 3 categories; endothelial NOS (eNOS or NOSIII), inducible NOS (iNOS or NOSII) and neuronal NOS (nNOS or NOSI) (Alderton et al., 2001). Of these, the most significant for microbes is iNOS. This form of NOS has been identified across a range of cells which can be stimulated, including vascular smooth, neutrophil and macrophage

cells, as well as glial cells of the central nervous system (Bogdan, 2001).

Because iNOS can only be found within the cytoplasm, it is necessary for diffusion to occur in order for NO to reach the phagosome and act upon the microorganisms within. In comparison with the production of $O_2^{\bullet -}$, NO is created at a later stage in the defence reaction, coming into play approximately 8 h after infection has occurred: this has been shown in studies using murine macrophages and *S. enterica* (Eriksson et al., 2003).

NOS comprises an amino-terminal oxidase domain which has a haem centre as well as sites to bind tetrahydrobiopterin and L-arginine. This domain connects with a calmodulin-binding domain which is small in length and leads to a carboxy-terminal reductase domain which has sites to bind to flavin mononucleotide (FMN), FAD and NADPH (Fig 1.2) (Stuehr, 1999).

NOS in its functional form is dimerised through incorporating haem, L-arginine and tetrahydrobiopterin. NADPH-derived electrons are transferred via NOS to FAD, on to FMN and then the neighbouring haem. Citrulline and $NO^{\bullet}$ are formed from oxygen and L-arginine via N^{ω}-hydroxy- L-arginine as an intermediate (reviewed in (Stuehr, 1999)) (Fig 1.2). Thus, overall nitric oxide synthases act as a catalyst in oxidizing one guanidine nitrogen within L-arginine to nitric oxide (NO) in the nitrosative burst. The hemoprotein takes as substrates dioxygen, as well as reducing equivalents which come from NADPH (Vazquez-Torres and Fang, 2001).

1.1.2.2 The Oxidative burst of phagocytic cells

Despite the comparatively inert nature of molecular oxygen, via enzymatic action, superoxide can be created, which is cytotoxic and can lead to the formation of oxygen radicals. In creating superoxide, professional phagocytes are dependent upon reducing equivalents produced by NADPH. This system is termed the phagocyte NADPH oxidase or respiratory burst oxidase (phox) system (Vazquez-Torres and Fang, 2001).

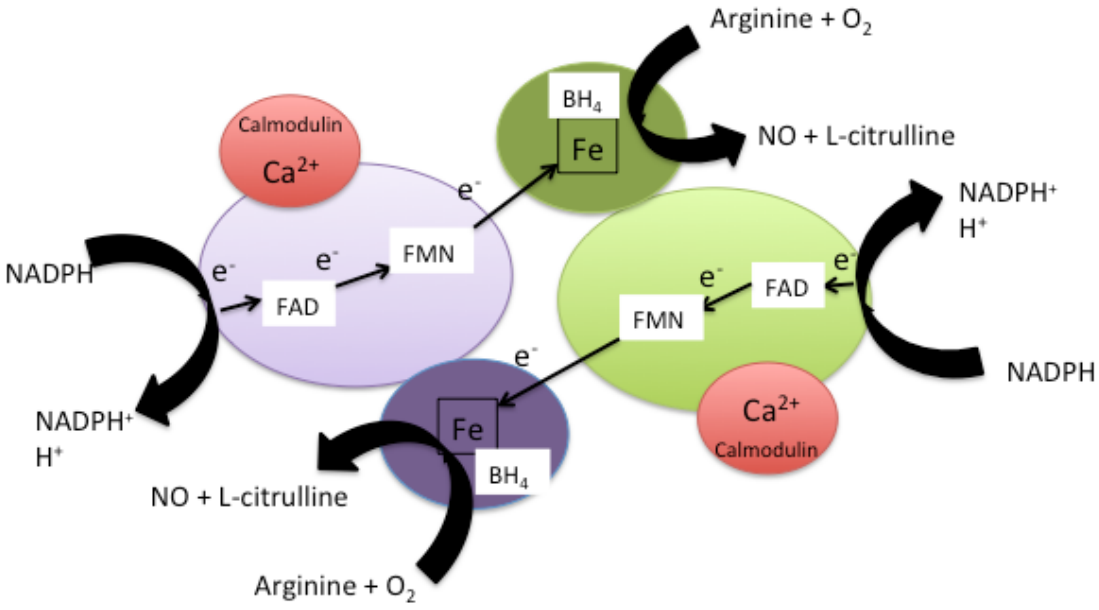


Figure 1.2 Transfer of electron within iNOS dimer. Electrons from NADPH electrons from the reductase domain are transferred first to flavin and then to an adjacent monomer's oxygenase domain haem. Adapted from (Alderton et al., 2001)

Many processes of the cell are prompted by both macrophage and neutrophil phagocytosis. The respiratory burst is one such action. In this process, the amount of oxygen taken up by the cell is increased, creating hypochlorous acid and hydroxyl radicals, which are both oxidants with powerful antimicrobial action (Knight, 2000). Reactive oxygen species (ROS) form an important part of the functions of the macrophage. Production of the multi-subunit NADPH-dependent phagocytic oxidase, known as Phox or NOX2, takes place on the phagolysosome membrane, and this allows electrons to be pushed into the compartment in order to produce superoxide anion $O_2^{\bullet -}$by reducing the oxygen (Slauch, 2011). This leads to the production of superoxide and H_2O_2 through the respiratory burst. While it was suspected for a long time that $O_2^{\bullet -}$ and H_2O_2 play a key role in the antibacterial activities of the macrophage, the exact role of H_2O_2 from the respiratory burst is now confirmed: this H_2O_2 is a secondary messenger, stimulating key signal pathways within alveolar macrophages to become active (Iles and Forman, 2002). NADPH oxidase activates the oxidative burst within a short time after phagocytosis. $O_2^{\bullet -}$ is reduced sequentially by one electron until hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), as other reactive oxygen intermediates, are formed.

The NADPH phagocyte oxidase complex crucially contains gp91-phox and p22-phox, which are proteins of the membrane, in addition to 3 cytosolic proteins, namely p47-phox, p67-phox and GTPase(s) Rac1 and Rac2 (Fig 1.3). Also, it is possible that the cytostolic p40-phox is implicated in regulation. Proteins gp91-phox and p22-phox make up the heterodimeric flavocytochrome b_{558} as described by Babior et al. (2002). Sites on the flavocytochrome are available to bind NADPH, flavin and 2 groups of haem. When Rac and p67-phox are present, there is electron transfer to FAD from NADPH and subsequently to gp91 haem centres, before transferring to oxygen and there forming $O_2^{\bullet -}$ (Fig. 1.3) (Fang, 2004) While the phagocyte membrane NADPH-oxidase system produces hydrogen peroxide in large quantities, macrophage and neutrophil cells in their activated state also release myeloperoxidase (MPO). This is bactericidal and fungicidal, owing to hypochlorous acid (HOCl), which is reactive in contact with superoxide anions, giving a hydroxyl radical ($^{\bullet}OH$) of high toxicity, as well as Cl^- (Vladimirov and Proskurnina, 2009).

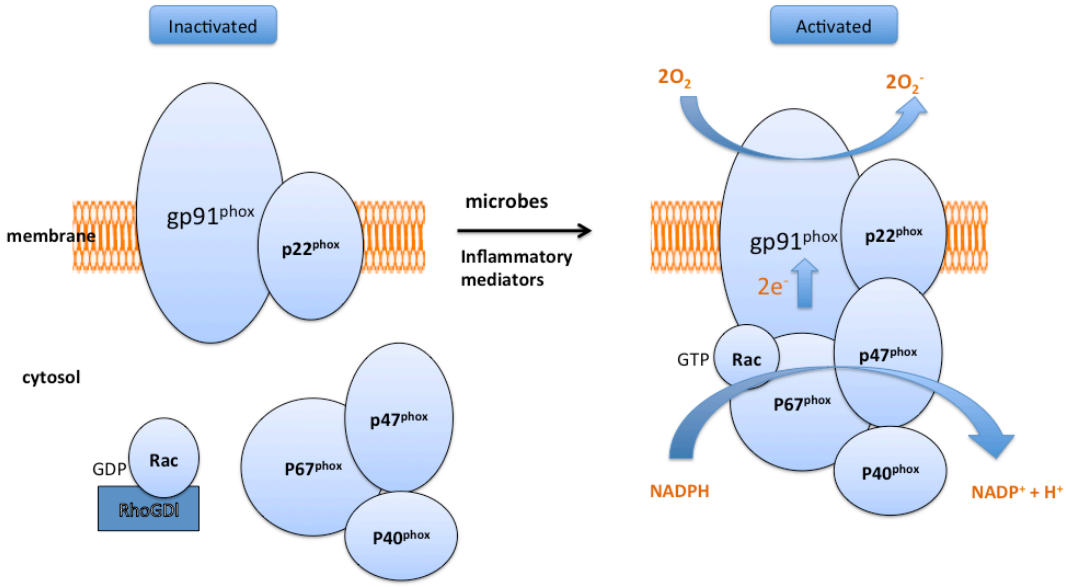


Figure 1.3 Phagocytic NADPH oxidase in states of rest and activation, in diagram form. Gp91-phox and p22-phox create a subunit for catalysis, flavocytochrome b558, associated with the membrane. Phox proteins p40, p47 and p67 create a cytosolic complex when the cell is in a resting state, translocating to the membrane when the cell is activated, and becoming docked to flavocytochrome b558. When the Rac G-protein binds to GDP, RhoGDI renders it stable during cell rest translocating to the membrane when the cell is activated. On assembly, enzymatic production of superoxide (O_2^-) ensues as electrons are transferred from NADPH in the cytoplasm to molecular oxygen (O_2). Adapted from (McCann and Roulston, 2013).

MPO release in activated neutrophils occurs from granules within the cell. The MPO enzyme is green, and at 5% of neutrophil dry mass, is the most frequent protein in such cells, with monocytes also containing it but in lesser proportions. As monocytes mature to become macrophages, their MPO productive ability declines. However, there is evidence that some cells which are similar to macrophages (including cells identified within atherosclerotic lesions) contain MPO (Davies, 2011).

1.1.2.3 The combined reactive species response

ROS and RNS function together, as shown through the example of murine macrophages which are immunodeficient in both iNOS and NAPDH oxidase (Phox): these macrophages lacked the ability to inhibit the spread of *Salmonella enterica* (Vazquez-Torres et al., 2000). Previously, Pacelli et al. (1995) had also shown a synergistic effect in killing *E. coli* with NO and H_2O_2. Superoxide $O_2^{\bullet-}$ and NO are capable of reacting together quickly and creating peroxynitrite ($ONOO^-$) (Fig 1.4), which is strongly toxic. While peroxynitrite is frequently considered to be the primary means of nitration of residues of tyrosine within proteins, *in vitro*, levels of nitrated tyrosine at pH 7.4 were lower where $O_2^{\bullet-}$ and NO were generated in tandem rather than by giving a bolus of $ONOO^-$ (reviewed in (Bowman *et al.,* 2011)).

1.2 Nitric oxide and reactive nitrogen species (RNS) in biology

Nitric oxide (NO) is a free radical gas which plays an important role in biological systems: especially in signalling and defence mechanisms. In mammals, NO at very low concentrations controls the blood pressure as reviewed by (Poole and Hughes, 2000). NO is a small and freely diffusible species. It is known as a toxic component of air pollution, and as a poison and ligand for haem proteins in physiology and biochemistry (Bowman et al., 2011).

NO is a small, neutrally-charged radical with an unpaired electron in an outer π orbital (Hughes, 2008). It is generated in a cellular environment at approximately 10^{-7} M, and it has a lifetime of about 30 min when it is oxidized to NO_2: therefore, NO has a short half-life under physiological conditions (Poole and Hughes, 2000).

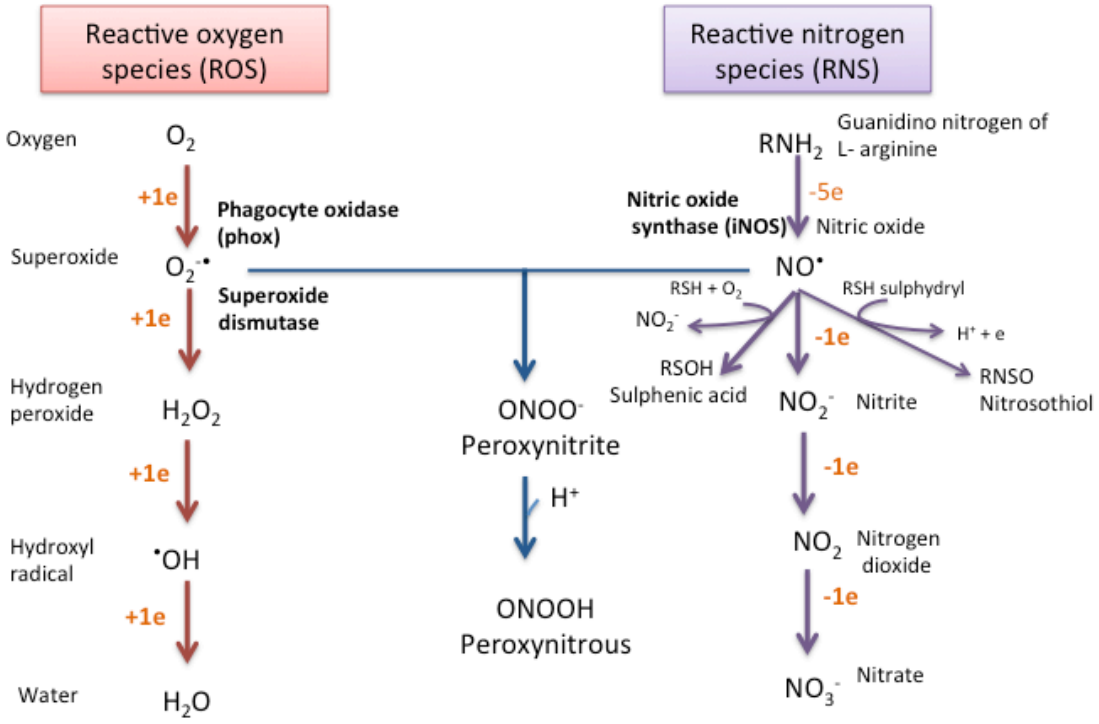


Figure 1.4 The production of nitrogen intermediates and reactive oxygen in mammalian cells. Nitroxyl anion (NO^-), which contains one electron and is a product of reduction of nitric oxide ($NO^{\bullet}$), does not commonly occur in normal conditions from NO•. Reactions between nitrogen species and cysteine sulphydryls may lead to S-nitrosylation, or to oxidation which creates sulphenic acid, in addition to the forming of disulphide-bonds. These processes can be reversed. Recognizable reaction patterns are identified for the peroxynitrite anion ($ONOO^-$) and for peroxynitrous acid (ONOOH). In the case of ONOOH, decomposition occurs spontaneously via a range of species similar to either hydroxyl (•OH) reactive radicals, or nitrogen dioxide ($NO^{\bullet}$), or both. Under limiting concentrations of L-arginine, the superoxide ($O_2^{\bullet-}$) and $NO^{\bullet}$ are produced by nitric oxide synthase, and this is favourable for peroxynitrite production. Adapted from (Fang, 2004).

NO also has a hydrophobic nature and small size, which makes it readily diffusible across cellular and lipid membranes; it therefore rapidly reaches and reacts with a diverse range of cellular targets (Pacher et al., 2007), and it does not react with water but is soluble in water (Poole and Hughes, 2000). In the cytoplasm, NO can react with haem (Hausladen et al., 2001), iron-sulfur (Fe-S) clusters (Cruz-Ramos et al., 2002) and thiols (Hess et al., 2005)

However, NO synthases in higher organism were reviewed earlier (section 1.1.2.1). NO synthases have been also identified in a number of bacterial species, and these enzymes show a number of similarities to those found in mammals. For example, they are also catalysts, producing NO through converting L-arginine firstly to N^{ω}-hydroxy-L-arginine (NOHA) and then NO. However, it is unclear in bacteria what the subsequent functions of this NO might be (Crane et al., 2010).

Endogonously-produced NO is suggested as a possibility for bacteria: especially where a species employs nitrite where oxygen is not present, as an electron acceptor. It is clear that NO accumulates in small concentrations within enteric bacterial cells, including *E. coli* when nitrite is reduced to form ammonia (Gilberthorpe and Poole, 2008). On the other hand, there is insufficient knowledge at present as to what function the creation of NO fulfils physiologically (Bowman et al., 2011). However, oxidative stress may be reduced and antibiotic resistance increased in gram-positive bacteria through producing NO, as it reduces the toxicity of oxidative stress (Gusarov et al., 2009).

$NO^{\bullet}$ molecules have a significant function within living organisms as antimicrobial effectors, and while research has focused on macrophage cells in producing $NO^{\bullet}$, it is possible that some forms of dendritic cells and non-phagocytic cells may utilize such molecules in combatting microbes (Bogdan et al., 2000). It should also be noted that while much $NO^{\bullet}$ is generated through enzymatic synthesis, bacteria in the mouth also reduce nitrate from food into nitrite, while gastric acid can subsequently convert this into RNS. This produces a significant barrier to the proliferation of pathogens in the intestines (Fang, 2004).

In biology, NO takes part in radical reactive processes which may protect or oxidise, and its reactions with different radicals could increase the toxicity and reactive properties (Hughes, 2008). Oxidation of radicals and the production of a number of poisonous substances such as the nitrosating agent nitrosonium (NO^+), nitrite (NO_2^-) and peroxynitrite ($ONOO^-$) are all intercellular products of NO (Poole and Hughes, 2000). NO reacts rapidly with many different targets, particularly: (i) iron centres (for example, the binding of NO activates guanylyl cyclase with the production of messenger cyclic GMP); (ii) thiols under oxidizing conditions; and (iii) with superoxide ions to give peroxynitrite ($ONOO^-$). Hence, the relation between the chemistry and the biological activity of NO is complex. NO can react in the cellular environment to produce new species with modified activity and functions (Poole and Hughes, 2000).

When NO and O_2 react together, nitrogen dioxide (NO_2) is produced (Equation 1).

$$2NO + O_2 \longrightarrow 2NO_2 \quad (1)$$

NO_2 oxidises a number of functional groups present in the biological context, including polyunsaturated fatty acids, cysteine thiols and tyrosines, for example. Where no reductants of NO_2 are present, the NO_2 produced through NO and O_2 reacting together will go on to form a reaction with more NO, as two radicals, producing dinitrogen trioxide (N_2O_3) as shown in Equation 2 (Bowman et al., 2011):

$$NO_2 + NO \rightleftharpoons N_2O_3 \quad (2)$$

The non-radical, electrophilic N_2O_3 reacts with H_2O, producing 2 NO_2 equivalents, as shown in Equation 3. In the presence of further nucleophiles (Nuc), such as amines or thiols, nitrosation of those nucleophiles takes place through N_2O_3 reacting with them, (see Equation 4) (Bowman et al., 2011).

$$N_2O_3 + H_2O \rightleftharpoons 2NO_2 + 2H^+ \quad (3)$$

$$N_2O_3 + Nuc \rightarrow Nuc - NO + NO_2 \quad (4)$$

Peroxynitrite ($ONOO^-$) is formed when NO reacts with superoxide ($O_2^{\bullet-}$) (Equation 5), and this can react in the cellular environment with carbon dioxide (CO_2) to form an intermediate (nitroperoxycarbonate) ($ONOOCO_2^-$) (Equation 6) (Hughes, 1999). $ONOOCO_2^-$ reduces to nitrogen dioxide (NO_2) and a carbonate radical ion (CO_3^-), which causes severe damage to cell contents. Peroxynitrite and other NO^- derivatives can induce a variety of changes in biological systems (Pacher et al., 2007).

$$NO + O_2^- \rightarrow ONOO^- \quad (5)$$

$$ONOO^- + CO_2 \rightarrow ONOO - C(O)O^- \quad (6)$$

The nitrosonium cation (NO^+) is the product of oxidation of nitric oxide, and may occur via interaction with metal salts or O_2. This species does not survive for long when in solution, quickly producing nitrous acid (HNO_2) on hydrolysation (Hughes, 1999). NO^+ has an important biological function due to the role that it is donor species in the process of nitrosation. This process requires the addition of the NO^+ group to nucleophiles such as thiols (generating S-nitrosothiols) and amines (generating N-nitrosamines) (Williams, 1999). NO does not act independently as an agent for nitrosation: rather, it plays this role if it is oxidised to form NO^+ in conditions of oxidation (Poole and Hughes, 2000). S-nitrosothiols and N-nitrosamines act as NO^+ donors, and thus nitrosating agents, and they are also reactive nitrogen intermediates (Hughes, 1999).

The nitroxyl anion (NO^-) is formed from the reduction of NO, which superoxide dismutase (SOD) and other agents of biological origin can cause (Murphy and Sies, 1991). Alternatively, Fe (II) within an acid solution can drive this reduction. Instability is shown by NO^- due to decomposing quickly, producing nitrous oxide (N_2O) on dimerising and dehydrating. $NO^{\bullet}$, NO^+ and NO^- are exchangeable *in vivo*; thus, all make a contribution to the biological functions of NO in nature (Hughes, 1999).

In a protein, when NO is transferred to metal centres, often occurring in iron sulfur and ferrous haem clusters, nitrosylation is said to occur (Hughes, 2008). However,

when a thiol or RSH is modified to *S*-nitrosothiol or RSNO, *S*-nitrosation occurs. Thus, NO may communicate and act as a signaling molecules at the intercellular and tissue level either directly or via its reaction products (NO^+, NO^- etc.) (Kettenhofen *et al.,* 2007).

1.3 NO as an antimicrobial molecule

NO's ability to combat microbial pathogens presents a high level of complexity. There are interactions between NO and the autoxidised products of NO and a range of targeted substances, such as residues of tyrosine, thiols, iron-sulfur, DNA bases and lipids. This allows NO to prevent the growth of a wide range of bacteria, both gram-positive and negative (Fang, 2004).

$NO^{\bullet}$ forms an essential part of innate immune responses against microbial pathogens, having a diffusive capacity through the membrane of the cell (Denicola et al., 1996), deactivating microbial enzymes (Ren et al., 2008), inhibiting respiratory function (Yu et al., 1997), and reacting with both O_2 and $O_2^{\bullet-}$, generating several reactive species of nitrogen, namely; $NO_2^{\bullet}$, N_2O_3, N_2O_4, and $ONOO^-$ (Fang, 2004).

Modification of iron-sulfur clusters [Fe-S] is carried out directly by $ONOO^-$ and NO, and this process results in the creation of nitrosyl-iron complexes (Pacher et al., 2007). Further, the findings of experimental work with microarray gene profiling demonstrate that genes involved in constructing and repairing clustered iron-sulfur were expressed at dramatically higher levels when *E. coli* cells were exposed to NO (Mukhopadhyay et al., 2004, Pullan et al., 2007, Hyduke et al., 2007). Taken together, this evidence points to proteins with iron-sulfur as a principle target for the cytotoxic properties of NO (Spiro, 2007).

Damage to DNA does not occur from NO itself, but from agents of nitrosation, which include N_2O_3, $ONOO^-$ and $NO_2^{\bullet}$, and can lead to DNA base nitration, causing breakage of strands and compromised ability for repair, which causes cross-links. In addition, $ONOO^-$ can cause DNA strands to break by targeting the sugar-phosphate DNA spine (Pacher et al., 2007). The amino acids tryptophan, histidine, cysteine and methionine can be oxidized by peroxynitrite (Pacher *et al.,* 2007). $ONOO^-$ is also capable of nitrating residues of tyrosine (Pacher et al., 2007).

The simplified diagram in Fig.1.5 indicates the biochemical reactions of NO in *E. coli* culture.

RNS can enter into reactions with lipids, DNA and other non-proteins. N_2O_3 and other species which are nitrosation agents have the direct ability for deamination of adenine, cytosine and guanine DNA bases, which respectively causes hypoxanthine, uracil and xanthine to be generated, and without repair will lead to transition mutations (Burney et al., 1999). Further, lipids show high vulnerability to NO_2, a mediator for peroxidization of lipids (Patel et al., 1999). Lipid peroxidation within the cell walls and membranes of bacteria can occur due to the presence of NO_2 and $ONOO^-$, rendering the membrane more fluid and permeable (Fang, 1997).

1.4 Oxygen and reactive oxygen species (ROS) in biology

Reactive oxygen species (ROS) represent a key element in the ability of macrophages to combat bacteria. Superoxide is produced by NADH-dependent phagocytic oxidase, and H_2O_2 is formed through dismutation (Slauch, 2011). Professional phagocytes use NADPH oxidase to synthesise significant amounts of superoxide ($O_2^{\bullet-}$) where infection is present. As mentioned in section 1.1.2.2, NADPH oxidase belongs to the Phox or Nox group of proteins which produce superoxide and are distributed in a range of tissues, with functions linked to the innate immune response outside of a phagosome context and also in signals transduction (Rada and Leto, 2008). The accidental production of superoxide ($O_2^{\bullet-}$) is possible where aerobic respiratory processes lead O_2 to be partially reduced by sections of the chain of electron transport: for *E. coli* a major source is NADH dehydrogenase II (Messner and Imlay, 1999). The amount of oxidative stress to which a microorganism is subjective is linked to the speed at which H_2O_2 and O_2 are formed (Imlay, 2003).

O_2 becomes more reactive when 1, 2 or 3 electrons join it, with 1 additional electron forming a superoxide radical ($O_2^{\bullet-}$), with 2 forming hydrogen peroxide (H_2O_2), and with 3 forming hydroxyl radicals ($^{\bullet}OH$). Alternatively, it can spin flip into singlet oxygen ($^1O_2$). Equation 7 shows how oxygen molecules are reduced to form water, with 4 electrons being added (Farr and Kogoma, 1991).

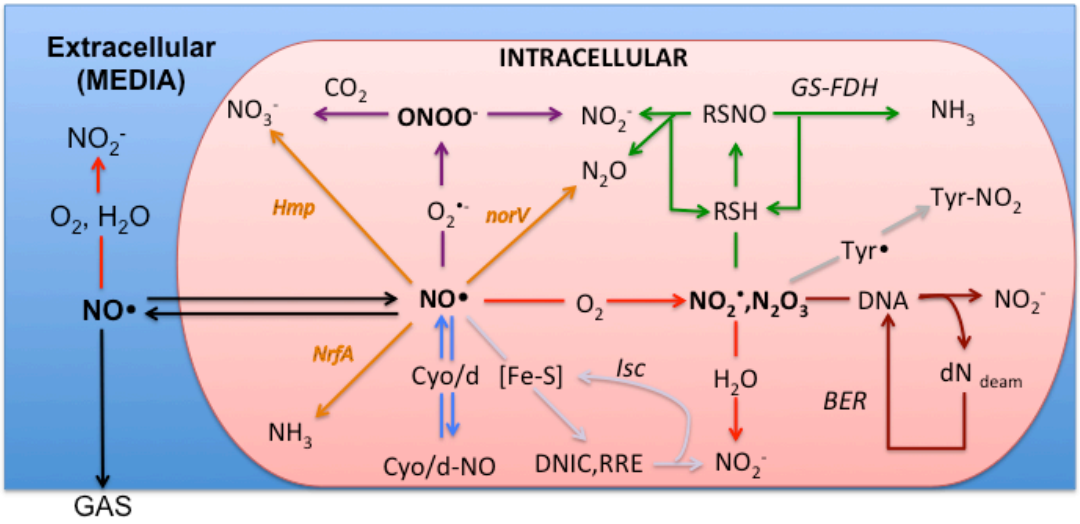
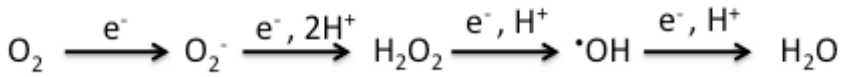


Figure 1.5 NO• network of biochemical reactions (simplified form) within cultured *E. coli*. Intracellular areas are shown in pink areas and outwith the cells in blue. Liquid media and gas phase are shown in contact with each other in the bottom-left of the diagram. NO• and oxidized reactive forms of NO•, including $NO_2^{\bullet}$, $ONOO^-$ and N_2O_3,) are given in bold, while enzymes or enzyme groups governing the pathway/reaction are given in italics. Autoxidation of NO• is shown via red arrows, while detoxification via enzymes is in orange, $ONOO^-$ as it forms and degrades is given in purple, inhibition of cytochromes in blue, iron-sulfur repair and nitrosylation in pale purple, nitrosation/denitrosation of thiol in green brown, nitration of tyrosine in grey, and repair and deamination of DNA in dark red. Adapted from (Robinson and Brynildsen, 2013).



(7)

$O_2^{\bullet-}$ shows different potential for reduction in different acidity and solution contexts. For solutions in water, its weak oxidising ability can lead thiols and ascorbic acid to be oxidised, in addition to its powerful reductive ability on ferric-EDTA and cytochrome c iron complexes. Through a dismutation reaction, $O_2^{\bullet-}$ undergoes additional transformations (Vatansever et al., 2013).

$O_2^{\bullet-}$, hydroxyl radical $^{\bullet}OH$ and hydrogen peroxide (H_2O_2) make up the reactive oxygen species (ROS). However, neither H_2O_2 nor singlet oxygen ($^1O_2$) can be classed as free radicals, although the other ROSs are (Lushchak, 2014, Ferrari *et al.,* 2011). Reactive oxygen species engage in direct reactions with cell/tissue/DNA carbohydrates, proteins, lipids and enzymes, and the resulting modifications can induce pathophysiologic conditions (Ferrari et al., 2011).

Harmful effects may be seen from the diffusion of H_2O_2 into the cytoplasm of the microbe. In fact, it is commonly asserted that it is the action on DNA which explains the effectiveness of phagocytic ROS in eliminating bacteria. For example, damage to DNA and aromatic amino acid auxotrophy is seen at ~0.5 μM cytoplasmic H_2O_2 in studies of *E. coli* (Slauch, 2011). $^{\bullet}OH$ is produced during reactions occurring between transition metals like ferrous iron and H_2O_2, in Fenton's reaction (Equation 8). This $^{\bullet}OH$ then undergoes further reactions with base and sugar molecules, and permanent damage is inflicted on the DNA by these reactions (Henle et al., 1999). As mentioned in Section 1.2, there is a possibility for nitric oxide to be combined with O_2^- to produce peroxynitrite, a substance with enhanced reactivity (Slauch, 2011).

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow {}^{\bullet}OH + {}^{-}OH + Fe^{3+} \qquad (8)$$

Hypochlorous acid (HOCl) is produced endogenously in immune cells as part of the oxidative burst response to infection, and is an antimicrobial compound due to its powerful oxidative effects (Gray et al., 2013). This acid is more widely called bleach, a disinfectant utilized on a global scale at a domestic level as well as in water treatment, hospital cleansing and where food is prepared or produced. HOC1 is distinct from H_2O_2, superoxide anions and redox-cycling pharmaceuticals in its

highly bactericidal effects and its high reactivity (Gebendorfer et al., 2012).

Myeloperoxidase, or MPO, is present in activated macrophage and neutrophil cells. As mentioned in section (1.1.2.2), this haem enzyme has bactericidal and fungicidal properties due to HOCl, which undergoes another reaction with a superoxide anion to produce Cl^- and $^{\bullet}OH$ as a hydroxyl radical with toxic effects (see Equation 9) (Vladimirov and Proskurnina, 2009). HOCl generates $^{\bullet}OH$ on reaction with Fe (II) (Fe^{2+})(see Equation 10), which substance can produce hydrogen peroxide (H_2O_2) on reacting with superoxide. Thus, each reaction produces substances with high toxicity and which have been demonstrated to be able to kill some intraphagosomal pathogens (Ferrari et al., 2011).

$$HOCl + O_2^{\bullet -} \rightarrow O_2 + Cl^- + {}^{\bullet}OH \qquad (9)$$

$$HOCl + Fe^{2+} \rightarrow Fe\,(III) + Cl^- + {}^{\bullet}OH \qquad (10)$$

MPO is important biologically, as shown through findings of research with individuals who are partially or totally deficient in MPO. In these cases, foreign bodies are generally phagocytosed in a normal manner by neutrophils, with long oxidative bursts generating $O_2^{\bullet -}$ and H_2O_2. However, the fact that MPO is insufficient raises the risk of having chronic infection (Davies, 2011). While MPO proteins are not in themselves effective bactericides, but react using enzymes with halide (Cl^-, Br^-, I^-) or pseudohalide (SCN^-) ions and H_2O_2 to produce hypohalous acids, which are oxidants. These include: hypobromous acid (HOBr), hypochlorous acid (HOCl), hypothio-cyanous acid (HOSCN) and hypoiodous acid (HOI). It is generally proposed that these acids form the major part of neutrophil action against bacteria, supported by other oxidising agents such as H_2O_2, nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{\bullet}$), as well as systems of enzymes, including lysosome, proteases and peptides (Davies, 2011).

1.5 ROS as antimicrobial species

Both RNS and ROS are capable of interacting with a range of targets inside the microbe, such as metal centres, thiols, lipids, protein tyrosines and nucleotide bases

(Nathan and Shiloh, 2000). Research with *E. coli* demonstrate that adverse impacts on DNA forms the central mechanism for antimicrobial effects based on ROS where there is a small concentration of H_2O_2 (Imlay and Linn, 1988). In contrast, at higher H_2O_2 levels, ROS mediates actions which harm a number of targets in the cell. Further, iron must be present for DNA to be damaged in this way, suggesting that the Fenton reaction takes place, producing the toxic ferryl or hydroxyl radicals as intermediates (McCormick et al., 1998). Where DNA bases are attacked oxidatively, they may generate products such as thymine glycol, hydroxymethyl urea and 8-hydroxyguanine. Meanwhile, where sugar is modified, breakage of strands may occur (Fang, 2004). Further, nucleobases may be oxidised, and mediation of this generally occurs as OH and carbon atoms containing a high density of electrons are added together. The result is that a broad range of bases with oxidative alterations can result from oxidation, and may be mutagenic if no repair is made (Cooke et al., 2003).

Proteins may also be oxidatively modified in different ways, and those proteins especially at risk of this include residues of methionine, cysteine, tyrosine, tryptophan and phenylalanine. Where lysine, proline or arginine is oxidatively modified protein carbonyls are found. Meanwhile, H_2O_2 introduced in *E. coli* resulted in the oxidation of proteins including enolase, alcohol dehydrogenase E, DnaK, elongation factor G, OppA, the F1-ATPase and OmpA (Fang, 2004). Oxidative action may mean that the polypeptide chain is cleaved, side chains of amino acids are modified and proteins altered to give derivatives with strong sensitivity to proteolytic degradation. Further, research demonstrates that peroxide is produced in large quantities where ROS acts on a range of amino acid residue forms, and particularly for those which are aromatic or involve sulfur in their construction. These include for example tyrosine, tryptophan, histidine, methionine and cysteine, in free states as well as on protein. Those peroxides may in turn produce intermediates which are reactive derivatives of protein carbonyl, which can harm a further range of targets, as for example in a cysteine thiol moiety (Vatansever et al., 2013). Lipid peroxidation in bacteria when ingested by a neutrophil has previously been demonstrated. There is reduced likelihood for peroxidation chain reactions to occur in bacterial membrane due to the existence of monounsaturated and saturated fatty acids. However, the lipids in the membrane may be significant within pathogenic eukaryotic species as areas harmed by oxidation (Fang, 2004).

Methionine, cysteine, glutathionine (γ-L-glutamyl-L-cysteinyl-glycine, GSH) and other compounds which contain sulfur react with HOCl 100-times faster than other cellular components. Sulfenyl chloride (R-SCl) intermediates are produced where cysteine thiols are oxidised with HOCl as a mediator of oxidation of cysteine thiols, but these are not stable and generate oxidised cysteine sulfenic acids or R-SOH on reacting with water. R-SOH intermediates are strongly reactive and subject to reduction by individuals from the thioredoxin group: alternatively, oxidation may be continued to form sulfinic acid ($R\text{-}SO_2H$) and sulfonic acid ($R\text{-}SO_3H$). These alterations to thiols generally cause proteins to degrade. Furthermore, cysteine sulfenic acids are readily reactive with different nearby cysteine thiols (R-SH), close proximity, generating reversible disulfide bonds (R-S-S-R`). While such bonds have relative stability, they may be further oxidised by HOCl, to give thiosulfonates [$R\text{-}S(O_2)\text{-}$ S-R`] or thiosulfinates [R-S(O)-S-R`]. Moreover, reaction of primary amines or secondary amines with sulfenyl chlorides leads sulfonamide linkages [$R\text{-}S(O_2)\text{-}$NH-R`] to be formed, and these are not reversible (Gray et al., 2013a).

Exposure to HOCl has long been observed to cause glucose respiration to rapidly decline, with AMP generated via conversion of the majority of ATP in the cell (Barrette et al., 1987). Further, the doses of HOCl is required for loss ATP, F1 ATPase inhibition, inability to replicate DNA, failure of protein transport and metabolite (Rosen et al., 1990, Rosen et al., 2009, Barrette et al., 1987). HOCl kills cells extremely quickly: first being exposed to HOCl, just 100 ms are required for fatal damage (Albrich and Hurst, 1982). Reactive chlorine species (RCS) may also kill cells by proteins crucial to the microbe being oxidatively unfolded and aggregated (Winter et al., 2008). Thus, bacteria lack of Hsp33 show accumulation of aggregated proteins and a sensitivity to HOCl treatment compare to wild-type strain (Gray et al., 2013a).

1.6 Bacterial response to RNS

1.6.1 Bacterial response to nitric oxide

A defence mechanism in pathogens against poisonous compounds is seen in terms of the production of a range of enzymes with the capability to detoxify these species (Baptista et al., 2012). In the pathogen *Escherichia coli*, at least three enzymes are

produced to counter NO; the best chractrized are flavohaemoglobin (Hmp), cytochrome *c* nitrite reductase (NrfA), and flavorubredoxin (FlRd), which is a flavodiiron protein and is encoded via the gene *norV* (Baptista et al., 2012). Hmp functions to a small extent as an NO reductase, but to a greater extent as a dioxygenase. Meanwhile, the periplasmic enzyme NrfA exhibits strong action as a NO reductase: however, the function of this enzyme *in vivo* is not fully established. Finally, FlRd appears to play a role in anaerobic conditions, where it scavenges NO (Baptista et al., 2012).

NsrR is a gene regulator which shows sensitivity to NO and nitrite (Bodenmiller and Spiro, 2006), and is concerned with those genes responsible for protecting cells from NO (Rankin et al., 2008). Analysis of the protein via microarray reveals its direct and indirect involvement in regulating over 30 genes, and several of these have involvement with protecting cells against RNS such as those which NO may produce (Filenko et al., 2007). NsrR falls within the Rrf2 group (Bodenmiller and Spiro, 2006), in which other members are IscR, a transcription factor which contains [2Fe-2S], as well as RirA, which regulates iron (Todd et al., 2002).

NO is detoxified by Hmp, a flavohaemoglobin. This occurs via a denitrosylase route which utilizes O_2 and generates NO_3^- in microaerobic and aerobic environments. Alternatively, it can occur at less rapidly without O_2 by reducing NO to N_2O (Poole, 2005b, Kim et al., 1999). Hmp's biochemical properties have been comprehensively described, being a bacterial globin through which NO may be bound at the haem ligand and it has been structurally analyzed to demonstrate binding domains for NAD(P) and FAD in the C-terminal section (Ilari et al., 2002). Electrons are possibly transferred from the NAD(P)H reductase domain by means of FAD to reach the ferric haem iron ligand, and in turn this liganded NO is reduced, creating an equivalent to a nitroxyl anion (NO^-) bound with haem (Fig 1.6) (Hausladen et al., 2001, Kim et al., 1999). Aerobically, NsrR is the main controller of Hmp, repressing expression when NO is not present (Fig 1.6) (Bodenmiller and Spiro, 2006).

Anaerobically, however, Fnr, is a global transcription regulator which responds to O_2 and bound to the *hmp* promoter, and thus expression is inhibited. In the presence of

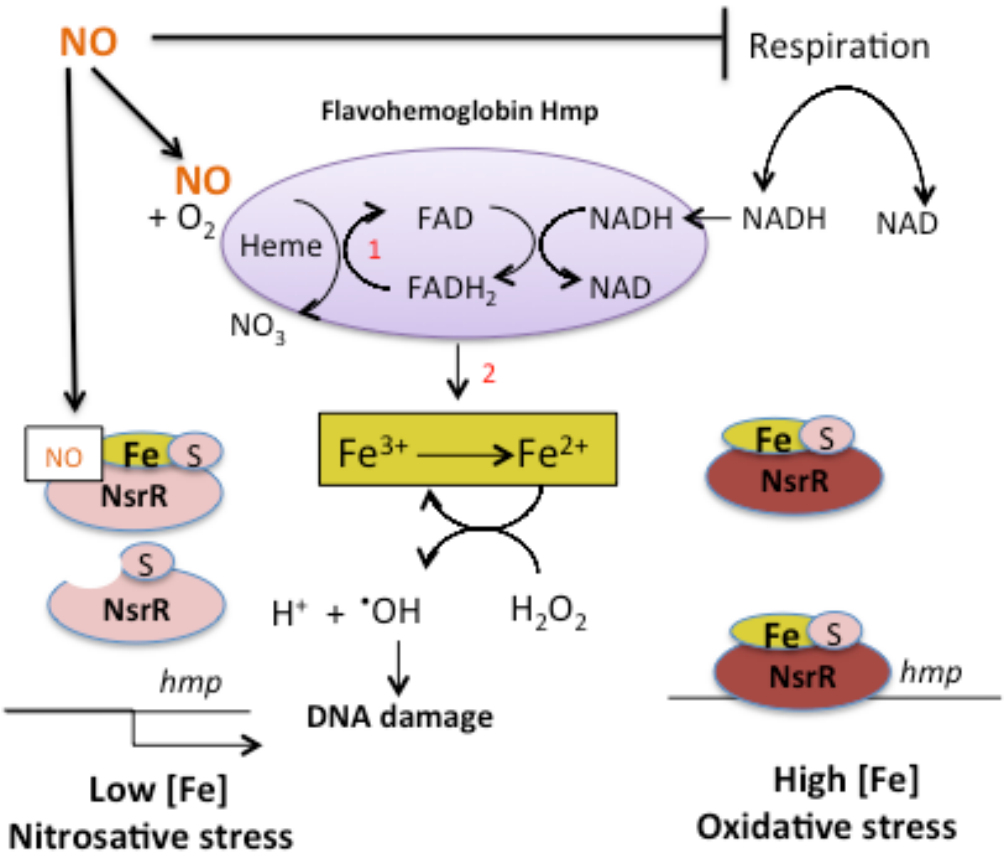


Figure 1.6 Hmp's role in maintaining homeostasis in redox. Hmp is derepressed through NsrR nitrosylation under nitrosative stress. NO is detoxified by Hmp, producing NO_3^- where O_2 is available and N_2O where it is not, and this reduces the damaging impact of NO on respiratory function. NAD(P)H donates electrons through FAD to haem, thus enabling either NO reductase or denitrosylation to occur (first pathway). Where NO is not present, flavin is reduced, driven by excess NAD(P)H, and this in turn reduces ferric iron (second pathway) and Fenton oxidative impact potentiation, meaning that NsrR expression of *hmp* is repressed where free iron levels are raised within the cell. Adapted from (Bang et al., 2006).

NO, (similarly to NsrR), Fnr's iron-sulfur cluster is subjected to nitrosylation. Its affinity with the *hmp* promoter is decreased and *hmp* transcription becomes derepressed (Cruz-Ramos et al., 2002, Poole et al., 1996). Moreover, Fur, which is the global regulator for over 90, *E. coli* genes, also has a repressive effect on *hmp* expression, when it binds to ferrous iron, which inhibits RNA polymerases in gene transcription of *hmp* and genes for uptake of iron. When the FeFur-NO complex is formed, DNA is unable to bind to Fur and *hmp* may therefore be transcribed (D'Autreaux et al., 2002). Further, substances such as sodium nitroprusside and GSNO donate NO^+ and stimulate the expression of *hmp* as homocysteine (a MetR co-regulator which has impact on the biosynthesis pathway for methionine) undergoes nitrosation. MetR in the presence of homocysteine activates *glyA* but the neighbouring *hmp,* which is transcribed differently, is repressed. When nitrosation depletes the pool of homocysteine however it is suggested that this will stimulate binding of MetR at a proximal location, initiating expression of *hmp* (Membrillo-Hernandez et al*.,* 1998).

NorR is a "NO reduction and detoxification Regulator," and forms part of a group of proteins with functions in regulation which respond to reactive species of nitrogen, including SoxR, OxyR and Fur. Transcription of *norVW* operon is activated by NorR in micro-aerobic and anaerobic environments, and this encodes a nitric oxide reducing flavorubredoxin for NO detoxification (Mukhopadhyay et al., 2004, Gardner et al., 2002). NorR was first described as a part of the response-regulating NtrC group (Pohlmann et al., 2000). Both *norR* and *norVW* have 3 NorR binding sites on their intergenic regions, and these use symmetrical 11-bp consensus sequences (Tucker et al., 2005), representing enhancer-binding locations for NorR to transcriptionally activate the genes (Tucker et al*.,* 2004).

In *E. coli* NorR activates divergent transcription of the *norVW* genes on exposure to nitroprusside, GSNO, acidified nitrite or NO. These genes, on activation, lead to the generation of a flavorubredoxin along with a partnering redox partner, and these allow reduction of NO to generate nitrous oxide, using NADH (Mukhopadhyay et al., 2004, Gardner et al., 2002). The multidomain NorV protein (flavorubredoxin, FlRd) contains an amino-terminal similar to β-lactamase module which has as its catalytic

centre a non-heme di-iron site, as well as a short chain module which is flavodoxin-like and an extension which is rubredoxin-like (Gomes et al., 2002). Small angle x-ray scattering produced an object at low resolution which demonstrated the independence of the activity of the rubredoxin domain (Petoukhov et al., 2008).

Research concerning *E. coli* and cellular global transcription responses under exposure to $NaNO_2$ and GSNO demonstrated that *E. coli* responded in a complex manner to reactive nitrogen species when grown aerobically within rich media, and further demonstrated the central part of Fur and NorR in that response, as well as lesser participation from OxyR and SoxR. It was further shown that other unknown regulators participate in this response. Interestingly, redox-active cysteines seem to be present in each of the four identified regulators (Mukhopadhyay et al., 2004).

The system comprising NorV/NorW involves nitric oxide reductase which reduces NO while oxidising NADH (Gomes *et al.,* 2002). The process of transferring electrons is started when NorW oxidises NADH, and an electron is transferred to NorV rubredoxin domain. The electrons move through the protein to reach the catalytic di-iron location and then participate in reducing NO, generating N_2O.

SoxR in *E. coli* was the first transcriptional regulator in bacteria which was found to give a NO response. This protein includes the cluster [2Fe–2S], and activation occurs when $[2Fe–2S]^{11}$ is oxidised by a single electron to form $[2Fe–2S]^{21}$. This is generally superoxide, based on the response of SoxRS to superoxide in oxidative environment (Pomposiello and Demple, 2001). On activation, SoxR allows *soxS* gene transcription synthesis of SoxS. *In vivo*, SoxR in its oxidised cluster undergoes reduction via a membrane- associated complex which is reliant on NAD(P)H, which, where superoxide is not present, may cause the system to return to a condition of rest (Koo et al., 2003). Experimental work *in vivo* and *in vitro* showed that purified SoxR may be activated by nitrosylation, which can mechanistically explain NO's impact upon the regulon of SoxRS. The SoxR species which is active on nitrosylation, has complexes of dinitrosyl-iron-dithiol. While it demonstrates comparative stability *in vitro,* disassembly or repair occurs rapidly *in vivo* where no NO is present (Fig 1.7 B) (Ding and Demple, 2000).

The gene *katG* is activated by OxyR and encodes hydro-peroxidase I switches on when exposed to S-nitroso-cysteine (Fig 1.8 B) (Hausladen et al., 1996). However, it is significant that neither NO nor nitrite had this impact when studied anaerobically *in vitro* (Hausladen et al., 1996). When purified OxyR from S-nitrosocysteine-exposed cells was obtained it was demonstrated to contain between 0.1 and 0.2 nitrosothiols per OxyR. Further, S-nitrosation of OxyR led to *katG* transcription *in vitro* (Hausladen et al., 1996). There is one cysteine within OxyR which is subject to a range of modification: nitrosation via S-nitrosothiols; hydroxylation on exposure to peroxide or air; and glutathionylation and derivatisation via the plant-based terpenoid avicins. Further, these changes each show a distinct result in terms of function in regulation (Kim et al., 2002). The picture presented thus contrasts with descriptions of OxyR which consider it a disulphide bond-mediated binary switch (Spiro, 2007). While S-nitrosylation exerts nitrosative stress and activates OxyR (Cys-199, S-NO), it is deactivated on de-nitrosylation (Cys-199, SH) (Fig 1.8 B) (Kim et al., 2002).

1.6.2 Bacterial response to $ONOO^-$

Peroxiredoxins are found across a broad range of systems in prokaryotes and eukaryotes, and frequently have an association with organic hydroperoxide and H_2O_2 reduction systems. Isolation of peroxiredoxin alkylhydroperoxide reductase subunit C (AhpC) from *Salmonella typhimurum*, allowed $ONOO^-$ to be broken down catalytically to form NO_2. The process first involves a cysteine residue being oxidised towards the protein's N-terminus. The flavoprotein AhpF reduces AhpC to activate catalytic turnover. The efficiency of catalysis was adequate for the protection of plasmid DNA against single-strand breakage (Bryk et al., 2000). A catalase-peroxidase function is detectable when peroxynitrite up-regulates the *katG* gene. This enzyme is known to function in the detoxification of peroxides, but it was also found that KatG is capable of expediting peroxynitrite breakdown (McLean et al., 2010a).

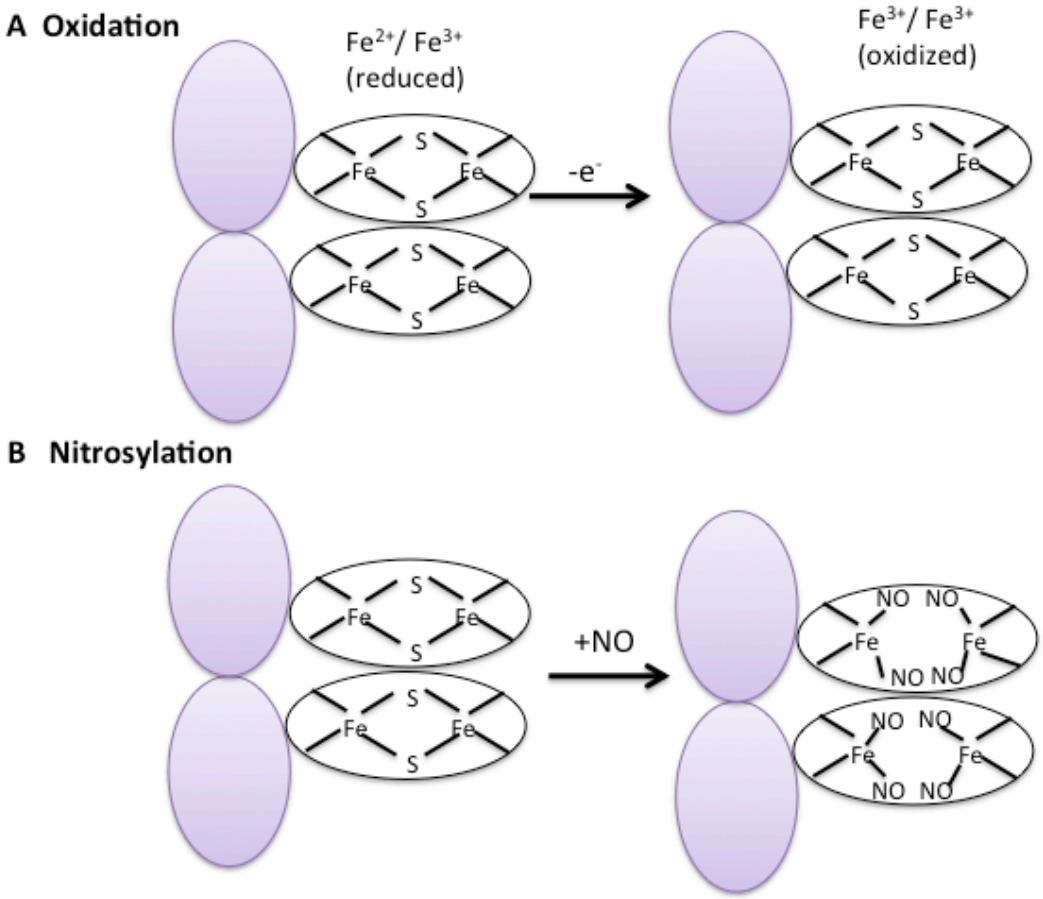


Figure 1.7 SoxR activation pathways. DNA-binding domains are given by shaded and iron-binding domains are given by unshaded ovals. SoxR is represented as homodimer. (A) Regulation by redox stress. Reduced SoxR [2Fe-2S] centres mean that soxS transcription is not activated at this stage. Transcription is activated when the protein is oxidized via a single electron. (B) Nitrosylation-based SoxR activation model. SoxR contains [2Fe-2S] clusters that undergo direct NO modification, producing dinitrosyl-iron-dithiol clusters, disrupting iron-sulfur clusters. Additional thiols are taken from SoxR protein's cysteine residues. Iron atoms are illustrated here as still protein-bound: however, this is not necessarily so, and especially where protein has been repurified. Adapted from (Ding and Demple, 2000)

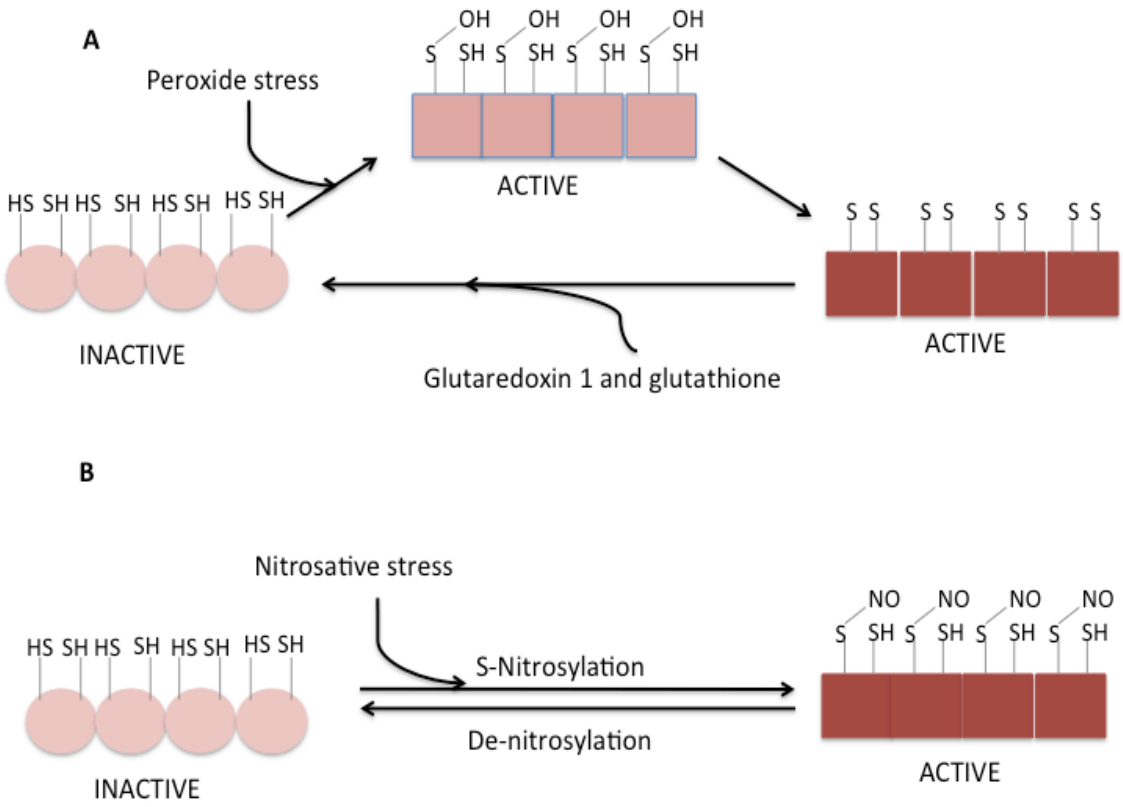


Figure 1.8 Outline of OxyR redox-reactivity. (A) All OxyR homotetramer monomers contain a C-terminal domain with sensor function, containing a cysteine residue (Cys-199) with reacts with redox agents to produce sulfenic acid (S-OH) when exposed to peroxide. It is possible that OxyR in this form regulates expression of genes, but more probably is an intermediate to the fully activated form which is bound intramolecularly between Cys-199 and Cys-208, with DNA-binding ability and transcription regulation function. Inactivation of OxyR (Cys-199, SH; Cys-208, SH) occurs via glutathione and glutaredoxin 1. (B) OxyR also presents a response to nitrosative stress. S-NO is formed when Cys-199 is nitrolysed, and this activates OxyR, while de-nitrosylation to generate SH inactivates it. Adapted from (Green et al., 2014).

1.7 Bacterial response to ROS

1.7.1 Bacterial response to $O_2^{\bullet-}$

The protein sensor SoxR recognises redox stress, with SoxS being a transcriptional activator involved in positive regulation of approximately 24 genes at chromosome level (Pomposiello et al., 2001). SoxR can be described as a homodimer, with the monomers each containing a cluster of redox-active [2Fe–2S], which can be oxidised and reduced (Fig 1.7 A) (Ding and Demple, 1997).

The SoxRS regulon contains genes which encode proteins such as superoxide dismutase (SOD), participating in detoxifying ROS, as well as endonuclease IV, repairing damage mediated by ROS involved in detoxification of ROS, and fumarase C, which can replace ROS-sensitive elements with resistance to ROS. In this way, toxicity from the oxidative burst initiated by macrophages reviewed in Green et al. (2014).

Frequently gram-negative bacterial species synthesise SOD isozymes (periplasmic/cytoplasmic), and these form the first protection to combat O_2^-. In *E. coli,* two SOD isozymes are found in the cytoplasm, with 1 being manganese-cofactored (MnSOD) and the other iron-cofactored (FeSOD). A further periplasmic enzyme is made of a copper zinc-cofactored enzyme (CuZnSOD, or SodC), It is challenging for O_2^- to pass through the membrane when pH conditions are neutral (Korshunov and Imlay, 2002).

1.7.2 Bacterial response to H_2O_2

In conditions of oxidative stress, firstly SoxR(S) and OxyR regulons are upregulated (Aussel et al., 2011) and then a number of additional proteins with a protective function are activated (Imlay, 2008).

OxyR belongs to the transcription factor group LysR, and organises responses under peroxide stress in a number of microbial species (reviewed in (Green et al., 2014)). OxyR is a homotetramer, and has 2 domains per subunit, one being N-terminal DNA-binding and the other C-terminal sensory. This second domain has cysteine residue

which react with redox species (Cys-199) (Choi et al., 2001). The OxyR transcription factor responds to oxidative stress, activating antioxidant gene expression when hydrogen peroxide is present in *E. coli* (Choi et al., 2001). H_2O_2 activates OxyR when Cys-208 and Cys-99 form an intramolecular disulfide bond. This probably occurs when Cys-199 is oxidised to an intermediate sulfenic acid (Fig 1.8 A). This acid then reacts with Cys-208, creating a stable disulfide bound which locks OxyR into an activated state. Oxidised OxyR is deactivated when this bond is reduced, and this allows OxyR to switch reversibly in the cell (Fig 1.8 A) (Zheng et al., 1998). To induce OxyR, diffusion of H_2O_2 within the bacterial cytoplasm must occur to ≤ 100 nM concentration (Slauch, 2011). Wide-ranging techniques have been utilised to identify several genes which are activated by OxyR. These include *katG,* which encodes hydroperoxidase I; *ahpCF,* which encodes an alkyl hydroperoxide reductase; *oxyS,* which encodes a minor regulatory RNA; *dps,* which encodes a nonspecific DNA binding protein; *gorA,* which encodes glutathione reductase; *grxA,* which encodes glutaredoxin 1; *trxC,* which encodes thioredoxin 2; *fur,* which encodes the Fur repressor of ferric ion uptake; and *dsbG,* which encodes a disulfide chaperone-isomerase (Zheng et al., 2001).

When H_2O_2 oxidises OxyR, a disulphide bound is created and changes the protein conformation, allowing improved bind by capabilities with *katG* promoter, for example (Zheng et al., 1998). To reduce the presence of H_2O_2 as far as possible, *E. coli* and other bacteria use hydroperoxidase II (HPII) as a single-function catalase, and also peroxidase- hydroperoxidase I (HPI) as a dual function catalase, with its other function being the breakdown of organic hydroperoxides. Catalase is the driver of the formation of H_2O and O_2 by converting H_2O_2, which occurs in 2 stages. Ferric haem is first oxidised by H_2O_2 to form compound I, which is an oxy-ferryl species (Equation 11). A further H_2O_2 molecule then reduces this compound I to its ferric species, and O_2 and H_2O are formed (Equation 12). This process is similarly followed with peroxidase, except that it is generally organic hydroperoxide which reduces compound I (Loewen et al., 2000).

$$\text{Haem} - \text{Fe}^{\text{III}} + \text{H}_2\text{O}_2 \rightarrow \text{Haem} - \text{Fe}^{\text{V}} = \text{O (Compound I)} \qquad (11)$$

$$\text{Haem} - \text{Fe}^{\text{V}} = \text{O} + \text{H}_2\text{O}_2 \rightarrow \text{Haem} - \text{Fe}^{\text{III}} + \text{H}_2\text{O} + \text{O}_2 \qquad (12)$$

Peroxiredoxin alkyl hydroperoxide reductase (AhpCF) is also present in *E. coli* and other bacteria, and also scavenges H_2O_2. Its activity with peroxide is located in the AhpC module, in which H_2O_2 is reduced via one cysteine residue, which acts as an oxidase for sulfenic acid. The species thus formed then bonds intramolecularly to a further cysteine residue and can then protect against being inactivated irreversibly through continued oxidation processes. The reductase partner AhpF regenerate the catalyst activity of AhpC by transfering an electron to the redox active disulphide centres from NADH by means of a flavin (Jonsson et al., 2007).

As with *katG*, *ahpCF*, expression undergoes positive regulation by OxyR when H_2O_2 is present. However, unlike KatG, low concentrations of H_2O_2 allow AhpCF to be affective, and this may be because this protein is highly efficient in scavenging this substance (Seaver and Imlay, 2001).

1.7.3 Bacterial response to HOCl

N-ethylmaleimide reductase or NemA forms part of the flavoprotein "old yellow enzyme" group. It is a catalyst for reducing NEM, or N-ethylmaleimide, to N-ethylsuccinimide (Miura et al., 1997). Cysteine residues of cell proteins are altered by NEM, with an inhibitory effect for growth. Further, it is considered that NemA may be significant in degrading compounds and residues which show toxicity and recycling these to obtain nitrogen (Umezawa et al., 2008).

The widely-conserved repressor of the TetR group, NemR in *E. coli*, was first characterised as sensing electrophiles capable of modification of cysteine. The work of Gray et al. (2013b) both *in vivo* and *in vitro*, demonstrated the usage of cysteine residues which have redox sensitivity are used by NemR in sensing *N*-chlorotaurine and HOCl. NemR was not found to RSN or ROS. Further, glyoxalase 1 (*gloA*) and *N*-ethylmaleimide reductase (*nemA*), which are enzymes for detoxifying electrophiles and are subject to regulation by NemR, are needed to survive exposure to HOCl. As HOCl reacts with others, *in vivo*, a range of electrophiles with toxicity can be formed.

NemR, or N-ethylmaleimide reductase repressor, is a regulator of the TetR type in *E. coli*. It is subject to Cys oxidation when it comes into contact with HOCl, leading to glyoxalase I (*gloA*) and N-ethylmaleimide reductase (*nemA*) to be derepressed. These have been demonstrated by microarray and quantitative RT-PCR and the study revealed that *nemR* was up-regulated 70- fold. Further, *gloA* was up-regulated to10-fold and *nemA* was also up-regulated 100-fold, where HOC1 was applied in less than lethal quantities. Within a *nemR* mutant strain, *nemA* and *gloA* did not respond to HOCl. This suggests that the expression of these genes is depended on NemR (Gray et al., 2013b).

Hsp33 is a conserved chaperone heat shock protein which is regulated by redox, and is activated through Cys oxidation and intrinsic unfolding on exposure to HOCl stress, inhibiting cell protein aggregation (Winter et al., 2008).

1.8 Scope of thesis

The innate immune system utilizes a plethora of weapons, with initial 'respiratory' bursts of ROS and RNS with which to fight internalized bacteria. Unsurprisingly, bacterial cells have the ability to cope and survive against ROS and RNS by generating proteins that can scavenge ROS and RNS and repair cellular damage caused by these toxic species. The work in this thesis aimed to mimic in some chemical aspects, the stresses encountered in an intracellular lifestyle in the model laboratory organism *E. coli* and the *hmp* mutant, in order to study the importance of Hmp as a defense against combined stresses. This thesis describes the impact of ROS and RNS on cell growth and viability under different experimental conditions to fully investigate the range of effects elicited by these species. The impact of combined stresses on the transcriptional changes that occur when bacterial cells are exposed to ROS and RNS, and the impact of the antimicrobial effects of NO combined with antibiotics was also investigated.

Chapter 2

Material and Methods

Chapter 2 Materials and Methods

2.1 Bacteriological methods

2.1.1 Strains

E. coli strains are described in Table 2.1

2.1.2 Media, agar and supplements

Sterilisation of the media and agar was achieved by autoclaving at 121 ºC and 15 p.s.i. for 15 min. All chemicals except where stated were obtained from Sigma, and sterilization of solutions and chemicals was achieved by filtering with 0.45 µm pore Millipore filters.

2.1.2.1 Luria Bertani broth (LB)

Tryptone 10 g, 5 g yeast extract (from Oxoid) and 10 g NaCl (Fisher) were dissolved in 1 L dH_2O and pH adjusted to 7.0

2.1.2.2 Nutrient agar (NA)

Nutrient agar (Oxoid) was dissolved at 2.8% (w/v) in dH_2O.

2.1.2.3 Evans medium

33 ml 1M Na_2HPO_4, 17.3 ml 1M NaH_2PO_4, 5 ml 2M KCL, 5ml 0.25 M $MgCl_2 \bullet 6H_2O$, 25 ml 4 M NH_4Cl, 5 ml 0.4 M Na_2SO_4, 0.38 g nitrilotriacetic acid, 5 ml 0.004 M $CaCl_2 \bullet H_2O$, 5 ml Trace Element, 10 µl of 3 mg / ml $Na_2SeO_3 \bullet 5\ H_2O$ and 20 ml 1M Glucose were dissolved together in 900 ml dH_2O. The pH was adjusted to reach 7.5 (Evans *et al.,* 1970) and the volume made up to 1 L.

2.1.2.4 Defined minimal medium

4 g K_2HPO_4, 1 g KH_2PO_4, 1 g NH_4Cl, 0.01 g $CaCl_2 \bullet H_2O$, 2.6 g K_2SO_4, 20 ml glucose (1M) and 10 ml trace element were added to 1 L of distilled water (dH_2O). Sterilisation was done by autoclaving

Table 2.1 *E. coli* strains

Strain	Genotype	Source/Reference
MG1655	Wild type *F*-lambda-*ilvG-rfb-50 rph-1*	Laboratory stock
RKP117	MG1655 *hmpA::*Tn5 Kan mutation transduced from RKP5867.	Laboratory stock
RKP2178	Δ (*argF-lacZ*)*U169* Φ(*hmp-lacZ*)	(Poole *et al.,* 1996)
RKP5917	Same as RKP2178 but *hmp* mutant	In this study
EC958	O25b:H4-ST131	(Totsika *et al.,* 2011)

2.1.2.5 Trace element

5 g Na_2EDTA was dissolved in ~ 800 ml dH_2O and the pH was adjusted to 7.4. Then, 0.5 g $FeCl_3$, 0.05 g ZnO, 0.01 g $CuCl_2.2H_2O$, 0.01 g $CoNO_3 \bullet 6H_2O$, 0.01 g H_3BO_3, 0.01 g ammonium and molybdate were added and made up to 1 L with dH_2O. The solution was filter sterilised and stored at 4 °C.

2.1.2.6 TB soft agar

Typtone (8 g) and NaCl (5 g) were dissolved in 1 L H_2O then pH was adjusted to 7.0 followed by adding 7 g of agar.

2.1.2.7 Phage lysate plates (PL)

2.5 g yeast extract, 4 g Tryptone, 2.5 g NaCl, 1 g glucose and 6 g agar were added to 500 ml dH_2O. The medium was sterilized by autoclaving then was cooled to 55°C and 5 ml $CaCl_2$ (0.5 M), 5 ml $MgSO_4$ (1 M) and 0.5 ml $FeCl_3$ (10 mM) were added. The plates were stored at 4 °C.

2.1.2.8 P1 plates

Yeast extract (2.5 g), Tryptone (4 g), NaCl (2.5 g), glucose (1 g) and agar (6 g) were dissolved in 500 ml dH_2O. The medium was sterilized by autoclaving then was cooled to 55°C and 5 ml $CaCl_2$ (0.5 M) was added. The plates were stored at 4 °C.

2.1.2.9 TY broth

Yeast extract (5 g), Typtone (8 g) and NaCl (5 g) were dissolved in 1 L dH_2O.

2.1.2.10 Antibiotic selection

Liquid agar and liquid media were supplemented with antibiotics at 50 ºC at 1/1000 dilution. The amounts added to make 1 ml stock solutions of antibiotics were as follows: Kanamycin (10 mg/ml) and Ampicillin (50 mg/ml). The stock solutions were prepared in dH_2O and filter sterilized using a 0.2 µm syringe filter and stored at -20ºC.

2.1.2.11 Strain storage

Strain stocks were stored at -80°C in LB containing 15% glycerol (v/v) for long-term storage while strains were cultured on NA plates and stored at 4 °C for periods up to 2 weeks for short time storage.

2.1.3 Culture conditions

2.1.3.1 Aerobic growth conditions

Starter cultures were prepared by inoculating 5 ml LB with a single colony of *E.* coli. LB medium was supplemented with antibiotics to maintain mutant strains carrying antibiotic resistance cassettes. Culture were grown overnight at 37 °C, 200 rpm. The secondary culture (1 % (v/v)) were prepared by inoculating the media with the starter culture and then incubated at 37 °C, 200 rpm.

2.1.3.2 Growth curve assays

The level of sensitivity to deferent concentrations of NO donors, H_2O_2 and HOCl in aerobic conditions was tested for *E. coli*. Cultures of 30 ml and 10 ml were contained within Klett flasks or conical flasks. Growth measurements were recorded every hour.

2.1.3.3 Culture turbidity measurements

The optical density measurements were conducted by using a Jenway 7305 spectrophotometer at 600 nm in cuvettes with a 1 cm path length against a medium blank or in a Klett Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) was used with filter 66 to determine the turbidity of the culture.

2.1.3.4 Cell viability assays

Samples from each culture was taken at measured times after stress and then diluted serially between 10^{-1} and 10^{-7} by using phosphate–buffered saline. 10 µl was taken from each dilution and placed onto nutrient agar by spotting. The plates were incubated overnight at 37 °C and colonies were counted. Then, calculation of means was made to determine colony forming units/ml.

2.2 Buffers and solutions

2.2.1 Phosphate-buffered saline (PBS)

A 10 x stock was prepared by adding 80 g NaCl, 14.4 g Na_2HPO_4, 2.4 g $KHPO_4$ and 2 g KCl in 1 L dH_2O and pH was adjusted to 7.4. Sterilisation was done by autoclaving. In order to obtain a 1 x PBS solution, the 10 x stock was diluted 10-fold by adding 100 ml 10 x to 900 ml dH_2O.

2.2.2 Phage dilution buffer

To prepare this buffer, 0.61g of Trisma base, 0.37 g $CaCl_2$ $2H_2O$, 1.23 g MgSO4 $7H_2O$, and 1.46 g NaCl were dissolved in 450 ml dH_2O and the pH was adjusted to 7.5 by using HCl. Then, the solution was made up to 500 ml.

2.2.3 Tris-HCl 50 mM, pH 7.4

7.88 g of Tris-HCl was dissolved in 700 ml dH_2O. The pH was adjusted and the solution made up to 1L (Sambrook and Russell, 2001).

2.2.4 Enzyme assay buffer

NaH_2PO_4 (5.4 g), Na_2HPO_4 (8.7 g), $MgSO_4$-$7H_2O$ (0.25 g) and KCl (0.75 g) were added to 1 L dH_2O and the pH adjusted to 7. The buffer was stored at 4 °C.

2.2.5 DTT (1 M) (dithiothreitol; Cleland's Reagent)

DTT (3 g) were added to 20 ml of 10 mM $NaCO_2CH_3$ (pH 5.2). The solution was filter sterilised and stored at - 20 °C.

2.2.6 Working buffer

40 µl of DTT (1 M) was added to 100 ml of enzyme assay buffer (section 2.2.4). The solution was prepared fresh and stored at 4 °C.

2.2.7 *o*-nitrophenyl-β-D-galactopyranoside (ONPG)

0.1 g of *o*-nitrophenyl-β-D-galactopyranoside was added to 25 ml of enzyme assay buffer (section 2.2.4). The solution was prepared fresh and stored at – 20°C.

2.2.8 β-galactosidase enzyme

β-galactosidase enzyme (Sigma) was dissolved in Tris HCl (10 mM), MgCl2 (10 mM) and 1 mM β-mercaptoethanol. The pH was adjusted to 7.3. The solution was prepared fresh and stored at – 20°C.

2.2.9 Reactive nitrogen species

2.2.9.1 Preparation and use of S-nitrosoglutathione (GSNO)

Ice and water was added to fill a 500 ml beaker, which was placed on a stirring plate. A foil-covered 100 ml conical flask with magnetic stirrer was then placed on the beaker. The entire equipment was located within a fume cupboard. L-gluthatione (reduced); 3.08 g was placed in the flask along with 18 ml of water at ice-cold temperature, in which the gluthatione dissolved. Following this, HCl (concentrated, 0.83 ml) and 0.69 g $NaNO_2$ were then placed in the solution, and it was then stirred for approximately 40 min, until the colour of the solution changed to pink. Following the addition of 20 ml ice-cold acetone, stirring was continued for an additional 10 min. Filtration of the solution was then conducted by means of a concentrator pump, and the GSNO remained on the paper as a pink solid. This precipitate was then washed with 2 ml ice-cold H_2O, and this was done five times, before washing three times with 10 ml ice-cold acetone and then washing a further three times using 10 ml diethyl ether. After this, a vacuum desiccator was applied overnight in limited light conditions to dry the precipitate (Hart, 1985). The GSNO solid was then kept at -70 ºC. A GSNO solution was created with a small amount of GSNO directly before its use in the experimental work. GSNO in solid state was placed in phosphate buffer at 55 ºC and dissolved. The absorption maxima of GSNO occur at 545 nm, and the extinction coefficient is 15.9 M^{-1} cm^{-1}.

2.2.9.2 DETA NONOate (NOC-18)

((Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate,3 (Enzo)) (25 mg). It was dissolved in 1 ml NaoH (0.1 M) to get a 153 mM stock and stored at -80 ºC protected from direct light. DETA NONOate has a half-life of 20 h at

37ºC and pH 7.4. It spontaneously liberates 2 equivalents of nitric oxide per mole (Table 2.2).

2.2.9.3 Inactivated DETA NONOate

DETA NONOate was made as described in section (2.2.9.2) and left at room temperature for 5 d. Then the solution was bubbled by nitrogen gas for 1 min.

2.2.9.4 NOC-5

(1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (Enzo)) (10 mg) was dissolved in 1 ml NaOH (0.1 M) to obtain a 56.75 mM stock and stored at -20 ºC protected from direct light. The half-life of NOC-5 at pH7 and 37 ºC is 25 min (Table 2.2).

2.2.9.5 NOC-7

(1-Hydroxy-2-oxo-3-(N-3-methyl-aminopropyl)-3-methyl-1-triazene (Enzo)) (10 mg) was dissolved in 1 ml NaOH (0.1 M) to obtain a 61.65 mM stock and stored at -20 ºC protected from direct light. The half-life of NOC-7 at pH7 and 37 ºC is 5 min (Table 2.2).

2.2.10 Reactive oxygen species

2.2.10.1 Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide was supplied at 30% (w/w) in H_2O and purchased from Sigma-Aldrich. The absorption maximum of this species was at 240 nm and using the extinction coefficient (43.6 M^{-1} cm^{-1}) stock concentrations could be determined. Solutions were used and stored at 4 ºC.

Table 2.2 NO-releasing compounds with the solubility of each compound and the half-life time (Aga and Hughes, 2008).

NO releaser	$t_{(1/2)}$ (min) 37ºC	$t_{(1/2)}$ (min) 22ºC	Solvent
DETA NONOate (NOC-18)	1260	3400	0.1M PBS (pH 7.4)
NOC-7	5	--	0.1M PBS (pH 7.4)
NOC-5	25	93	0.1M PBS (pH 7.4)

2.2.10.2 Hypochlorous acid (HOCl)

A solution of hypochlorous acid (HOCl) was prepared in 0.01 M NaOH by adding sodium hypochlorite (NaOCl). The pH was adjusted to 7.0 and the absorption maximum of this species was at 292 nm. An extinction coefficient of 350 M^{-1} cm^{-1} was used (Halliwell and Gutteridge, 1998). Solutions were prepared fresh before the experiment.

2.3 β-galactosidase assay

The β-galactosidase assay was preformed by growing bacterial cultures at 37ºC /200 rpm to mid-exponential phase (OD_{600} 0.4-0.7). Cell pellets were resuspended in 4 ml working buffer. The cell suspensions were stored on ice. Then, 10 µl of $CHCl_3$ and 5 µl of 0.1% sodium dodecyl sulphate (SDS) were added to permeabilize cells assay. The mixtures were vortexed for 10 s and incubated at room temperature for 5 min. The reactions were started by adding 200 µl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and the time of the reactions were recorded. The optical density at 420, 550 nm was measured for the reaction and the optical density at 600 was measured for the cell suspension (Poole *et al.,* 1996). Activities were calculated by using the formula of Miller as following :

$$\text{Units} = 1000 \bullet [OD_{420} - (1.75 \bullet OD_{550})] \div [t \bullet V \bullet OD_{600}]$$

t = time of reaction and V= volume of cell suspension in assay.

2.4 Molecular biology methods

2.4.1 Agarose gel electrophoresis

Agarose gel electrophoresis was conducted to view DNA fragments. DNA was typically resolved in agarose (1 g in 100 ml 1 x TAE, dissolved through heating). Ethidium bromide solution (Bio-Rad) (4 µl) was added to 50 ml agarose before pouring. DNA samples were typically loaded along with HyperLadder IV (BIOLINE) DNA ladder, submerged in 1 x TAE buffer and run for ~1 h at 100 V. Gels were subsequently visualised under UV light using the GeneGenius Gel Imaging System (Syngene).

2.4.2 Generalization transduction with bacteriophage P1 *vir*

2.4.2.1 Preparation of lysates

Lysates were made as described in (Miller, 1972). The donor cells (MG1655 *hmp* mutant) were grow overnight in TY medium contain 5 mM $CaCl_2$. P1 *vir* stocks were diluted in phage dilution buffer from 108 to 103 PFU/ml. Each dilution was mixed with 100 µl of the culture then incubated at 37ºC for 20 min. 1 ml of pre-warmed (37ºC) TB and 1.5 ml of warm (55ºC) TB soft agar were added to the phage/cell mix and then poured onto a phage lysate plate. The plates were incubated at 37ºC in moist atmosphere (wet box). The plates were incubated for 4 h or until a lacy was seen. Then, the plates were chilled at 4ºC for 30 min. An overlay of 5 ml of cold phage dilution buffer was added. The plates were incubated at 4ºC overnight and the overlay was harvested with a Pasteur pipette. Lysates were stored at 4ºC and a few drops of chloroform were added for long storage.

2.4.2.2 Generalized transduction

A culture of recipient cells (Φ(*hmp-lacZ*)) were grown at 37ºC in 2.5 ml TY supplemented with 5 mM $CaCl_2$. 100 µl of P1 *vir* stock from a lysate prepration were mixed with the same volume of recipient cells and then incubated at 37ºC for 20 min. The mixture was spread onto P1 plates supplemented with antibiotic and 0.125 mM of $Na_4P_2O_7$ and then incubated at 37ºC overnight. The potential transductants were re-plated onto selective media and the phenotype confirmed after overnight incubation.

2.4.3 Culture sampling and RNA stabilization

E.coli MG1655 wild type was grown in batch culture. Once the OD reached 0.4, DETA NONoate (0.3 mM), H_2O_2 (2 mM) and HOCl (50 µM) were added to the culture. A 5 ml volume were withdrawn from the batch culture as a sample after 10 min and 25 min. The culture samples were immediately mixed with 10 ml RNAprotect bacterial reagent (Qiagen) and shaken vigorously for 5 s. Then, the

mixture was incubated at room temperature for 5 min. After the incubation, the mixture was pelleted by centrifugation at 5000 x g for 10 min. Supernatants were discarded and pellets were stored at -80 ºC.

2.4.4 RNA isolation and purification

RNA was purified from cell pellets using an RNeasy mini kit (Qiagen) and following the manufacturer's handbook instructions. To enhance cell lysis, TE buffer containing 10 mg/ml lysozyme was used. The RNA samples were stored at -80°C.

2.4.5 RNA validation

The concentrations of RNA in samples were determined by using a Nanodrop spectrophotometer (Thermo Fisher Scientific) with RNase free water as a blank. The Nanodrop was programmed to calculate RNA concentrations on the basis that one A_{260} unit of RNA corresponds to a concentration of 40 µg/ml. In order to estimate the purity of RNA samples, a ratio was determined at 260 nm and 280 nm. Samples with ratio ~ 1.8 were regarded as pure without contamination (Sambrook and Russell, 2001). A 1.25% agarose gel was used in order to test the RNA integrity. The presence of 2 distinct bands corresponds to the 16S and 23S rRNA and that showed the stability of the RNA sample.

2.4.6 Primer design

Primers were designed for real time PCR experiments using Primer 3 software (v.0.4.0). Each primer was created to be 20-24 bp in length with a melting temperature (Tm) of 57-63 ºC and GC contents of 28-80% and generate a product 50-150 bp. Optimal primer pairs were selected on the basis that they exhibited similar melting temperatures, GC% and lacked any secondary structure. Primers were ordered from Sigma-Adrich and were re-suspended in super-pure H_2O to a final concentration of 500 µM. Table 2.3 indicates the sequence of the primers that used for real time PCR experiment.

Table 2.3 Primers utilized in RT-PCR experiments.

	Gene	Function	Primers
1	*katG*	Catalase-hydroperoxidase	F CGATATCCATAACACCACAGCC R TGGTTTAACAGGTCAACACGA
2	*ahpC*	Alkyl hydroperoxide reductase	F CCCGGCTGACTTTACTTTCG R CTTCGTAGTGGTCAGCAACG
3	*sodA*	Manganese superoxide dismutase	F TGAGCTATACCCTGCCATCC R TCTGATGGTGTTTGGTGTGG
4	*nemA*	N-ethylmaleimide reductase	F TCTCTGCGCGATGAAAATGG R AGATCAAAACCGGCTTCACG
5	*hmp*	Flavohaemoglobin	F CGCTCAAACCATCGCTACAG R GGTTAACTTTGGCCCCGTTT
6	*norV*	Nitric oxide reductase	F AATTCCCGATACGCCGATCT R GTCGCCAGTTTTCACCACAT
7	*gyrA*	DNA gyrase	F GCGACCTGCGAGAGAAATT R CATCTCGGACATCTGGCAGC

2.4.7 Primer pair verification

PCR was conducted using genomic DNA as a template in order to determine whether primer pairs were able to drive the synthesis of selected genes (Tables 2.3 for primer sequences). The reaction mixture contained of 2 µl DNA, 1 µl forward and reverse primer (10 µM), 25 µl Dream taq Green Master Mix (2x) [Ferments] and was made up to 50 µl with nuclease-free water. The reaction was subsequently subjected to the following thermal cycle:

95 ºC	5 min	1 cycle
95 ºC	30 s	25 cycles
62 ºC	2 min	
72 ºC	2 min	
72 ºC	8 min	1 cycle

PCR products were run out on 2 % Agrose gel electrophoresis (section 2.4.1). Primer pairs that gave a single PCR product were selected for use in the next stage of RT-PCR.

2.4.8 Genomic DNA extraction

Genomic DNA was purified using Qiagen's DNeasy Blood and Tissue kit and following the manufacturer's handbook instructions. DNA concentrations were determined photometrically by measuring absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

2.4.9 Real Time PCR

RT- PCR experiment was conducted in 96-well polypropylene reaction plate (Agilent). Genomic DNA at 20 ng/µl concentration was serially diluted two fold to 0.04 ng/µl and 5 µl of each concentration was added to separate column on the plate. A 2x Brilliant III SYBR Green qRT-PCR MasterMix (Sigma) (10 µl), forward primer (1.25 µl), reverse primer (1.25 µl), 100 mM DTT (0.2 µl), RT/RNase block (1 µl) and 1.3 µl nuclease free H_2O were mixed and 15 µl was then added to each well. The 96-

well plate was placed into the Mx3005P real time PCR machine (Stratagene). RT-PCR was carried out using the following settings:

50 ºC 10 min 95 ºC 3 min	}	1 cycle
95 ºC 15 s 60 ºC 20 s	}	40 cycles
95 ºC 1 min 55 ºC 30 s 95 ºC 30 s	}	1 cycle

Mx3005P real-time PCR system was utilized to measure gene expression through quantification of cDNA converted from initial mRNA template. This system includes a 96-well thermal cycler and a quartz tungsten halogen lamp that applies scanning optics for optimal recognition between single photomultiplier tube and samples. Light is directed across each well to trigger the SYBR Green fluorophore in PCR solutions,and fluorescence is collected by a photomultiplier tube. Eventually, a digital draft is sent to the computer.

2.5 Determination of NO by using NO electrode

NO detection was determined by using an NO electrode (Precision Instruments ISO NOP sensor (2-mm diameter)). The NO electrode was calibrated as described in the manufacturer's handbook (Corker and Poole, 2003). Evans medium was added to the chamber and supplemented with 25 μM NOC-7. H_2O_2 (250 μM) or HOCl (6 μM) were added to the Evans medium before or after NOC-7 addition.

2.6 Determination of minimum inhibitory concentrations (MICs)

In order to determine the minimum inhibitory concentrations (MICs) of antibiotics or NO donor against EC958 and MG1655, experiments were conducted in 96-well plates. Each compound was diluted in several concentrations in dH_2O. Four different antibiotics (doxycycline, cefotaxime, gentamicin, polymyxin B) were used. All the antibiotics were provided by Sigma and stored at 4 °C. Fresh stocks of antibiotics were prepared before use in experiments. The minimum inhibitory concentrations

(MICs) were determined by measuring the optical densities of the growth inhibition after 24 h incubation period. The minimum inhibitory concentration (MIC) is the lowest concentration where no growth is visible after a 24 h incubation period.

2.7 Checkerboard experiments

Checkerboard experiments were conducted as described in Orhan et al. (2005). Experiments were preformed in 96-well plates. A 200 µl of defined minimal medium (DMM) was distributed into each well and serial twofold dilutions of each compound were prepared. The first compound of the combination was serially diluted along the y-axis, while the second compound was diluted along the x-axis (Fig. 2.1). The plate was incubated in the plate reader (TECAN sunrise with Magellan software) at 37 °C for 24 h. The MIC and the combined MIC were determined and the combined effects were analyzed by a fractional inhibitory concentration (FIC) index. FIC was calculated as the MIC of first compound and second compound in combination, divided by the MIC of the first or the second alone. Then, the FIC index was determined by adding the FICs. The following formula interprets the ΣFIC:

$$FIC_{\text{compound 1}} = \frac{MIC_{\text{compound 1}} \text{ in combination}}{MIC_{\text{compound 1}} \text{ alone}}$$

$$FIC_{\text{compound 2}} = \frac{MIC_{\text{compound 2}} \text{ in combination}}{MIC_{\text{compound 2}} \text{ alone}}$$

$$\Sigma FIC = FIC_{\text{compound 1}} + FIC_{\text{compound 2}}$$

When ΣFIC is ≤ 0.5, this indicates a synergistic effect while ΣFIC > 4 shows antagonistic effect. On the other hand, 0.5 < ΣFIC ≤ 4 of the two compounds shows indifferent or additive effects (Chung *et al.,* 2011).

1	1	2	3	4	5	6	7	8	9
A	Growth control	1/32 MIC	1/16 MIC	1/8 MIC	1/4 MIC	1/2 MIC	MIC for compound 1	2x MIC	4x MIC
B	1/8 MIC for compound 2								
C	1/4 MIC for compound 2								
D	1/2 MIC for compound 2								
E	MIC for compound 2								
F	2x MIC for compound 2								
G	4x MIC for compound 2								
H	8x MIC for compound 2								

Figure 2.1 Checkerboard method indicting the synergy of two compounds in combination. The grey cells shows growth and unfilled cells shows no growth.

Chapter 3

Enterobacterial recovery from concerted nitrosative and oxidative stresses

Chapter 3. Enterobacterial recovery from concerted nitrosative and oxidative stresses

3.1. Introduction

Phagocytic cells including macrophages and neutrophils are one of the most important and first host defense lines of the innate immune response in mammalian cells. These phagocytic cells assault microorganisms and use reactive oxygen, nitrogen and chlorine species for host resistance to microbial pathogens (Fang, 2004). During the course of innate immunity, free radicals from phagocytic cells are important to kill human pathogens. Bacterial pathogens avoid these stresses (oxidative/nitrosative burst) by using antioxidant genes and enzymes (Ferrari et al., 2011).

Phagocytic cells contain two antimicrobial systems. The first system is NADPH phagocyte oxidase (phox) which generates superoxide ($O_2^{-\bullet}$) and the second is inducible nitric oxide synthase (iNOS), which is responsible for nitric oxide ($NO^{\bullet}$) generation (Fang, 2004). Half of the superoxide reacts with H^+ to produce hydrogen peroxide reviewed in Ferrari et al. (2011). In addition to the NADPH phagocyte oxidase (phox) system, activated neutrophils and macrophages contain myeloperoxidase (MPO), which generate hypochlorous acid. HOCl can react with superoxide anions to generate hydroxyl radical ($\bullet OH$) and Cl^- (Ferrari et al., 2011). To detoxify superoxide and hydrogen peroxide, bacterial pathogens have several enzymes, such as superoxide dismutases or reductases, catalases and peroxidases (Imlay, 2008), while NO dioxygenases and reductases are the NO scavengers in pathogenic bacteria (Bowman et al., 2011).

Despite well-studied protective measures mounted by the pathogen to resist these onslaughts, the vast majority of bacteria are killed by this offensive strategy, but others do manage to survive and multiply within macrophages. It has been shown that human primary macrophages kill about 20% of intracellular *S. Typhi* after 2 h of incubation and >90% by 24 h (Forest et al., 2010); thereafter the numbers of intracellular `persisters` remains stable. The outcome of exposure of a host to *S.*

Typhi depends on the effectiveness of the macrophage response and the counter-responses of the bacterium.

A further example of how bacteria recover from stress is in stationary phase of batch cultures. Rolfe et al. (2012) observed that when 2-day old bacterial cells in the stationary phase are placed in a new environment, within four minutes the transcriptional machinery mediates significant alterations in physiology. This process represents an adaptation phase connecting the transcriptional programmes for the stationary and lag phase. The high expression of genes involved in iron uptake, Fe-S cluster synthesis and manganese uptake during the lag phase period confirm the important role that these metals play in lag phase period to help bacteria cells prepare for exponential phase. The study also revealed that *Salmonella* viability becomes more sensitive to H_2O_2 at 4 min and 20 min of lag phase period and that related to the combination of intracellular iron and oxygen, which increases the Fenton chemistry and make the cells more sensitive.

Doerr et al. (2010) suggested that when pathogens are stressed by different host-related stress (high temperature, oxidants, low pH, membrane-acting agents), these cells try to deploy two lines of defence: (i) activate stressed related genes in order to be resistance; (ii) transform part of the bacterial cell population into a dormant state as a way to survive from stress via an inactivated state.

The work in this chapter aimed to mimic the stresses encountered in an intracellular lifestyle in the model laboratory organism *E. coli*. We will seek conditions that result in killing of approximately 90 % of the population and then study the response of the survivors in growing and increasing viability and growth after the stresses. In this work, a *hmp* mutant was used alongside wild type *E. coli* as a system for comparison, in order to study the importance of *hmp* as the major defense against combined stresses that include nitrosative stress.

3.2 Results

3.2.1 Effects of GSNO on bacterial cells growth and viability

GSNO (*S*-nitrosoglutathione) reacts with cysteine and homo-cysteine followed by disruption of methionine biosynthesis pathway which affects the metabolic and regulatory systems in *E. coli.* The reaction of GSNO with cysteine and homo-cysteines cause a significant changes in regulatory activity of MetR, MetJ and CysB and lead to growth inhibition (Jarboe et al., 2008). In addition, GSNO cause an up-regulation to the genes response to NO such as *hmp* and *norV* (Flatley et al., 2005, Membrillo-Hernandez et al., 1998).

In order to establish the effects of GSNO (*S*-nitrosoglutathione) on the growth and viability of *E. coli* wild type and *hmp* mutant strains and determine the concentration of GSNO capable of killing 90% of bacterial cells, *E. coli* wild-type and *hmp* strain were grown in 2 ml Evans medium and GSNO (*S*-nitrosoglutathione) was used as nitrosative stress at different concentrations (0, 0.3, 0.5, 1, 3, 5 mM). After overnight incubation, the optical density was recorded at 600 nm to demonstrate the effect of GSNO on the growth of both the *hmp* mutant and wild type strains (Fig. 3.1). Figure 3.1 shows that the *hmp* strain presents a significant reduction in growth when these cells are exposed to 1 mM GSNO and higher. The *hmp* mutant strain was more susceptible to GSNO than WT due to the consumption of nitric oxide by the flavohemoglobin (Hmp) enzyme in the wild type strain.

The growth curve was complemented with viability assays to establish whether 1 mM GSNO would decrease the bacterial cells counts. The *hmp* strain was grown in 30 ml of Evans media supplemented with kanamycin (KM) and inoculated to 5 % (v/v). GSNO was added at 30 Klett units at different concentrations (0, 0.3, 0.5, 0.6, 0.8 and 1 mM). The growth of bacteria was measured every h (Fig 3.2 A). The viable count was assessed after various periods of time (0, 3 and 5 h) (Fig 3.2 B). This work found that 1 mM GSNO was enough to stop the growth of the *hmp* mutant strain. Moreover, 0.3, 0.5 and 0.6 mM of GSNO caused a significant reduction in the growth of the *hmp* mutant strain. The viability of the cells (Fig 3.2 B) shows that 0.3 and 0.6 mM of GSNO caused approximately 70 % reduction in the cells viability after 3 h of GSNO treatment compared to untreated cells.

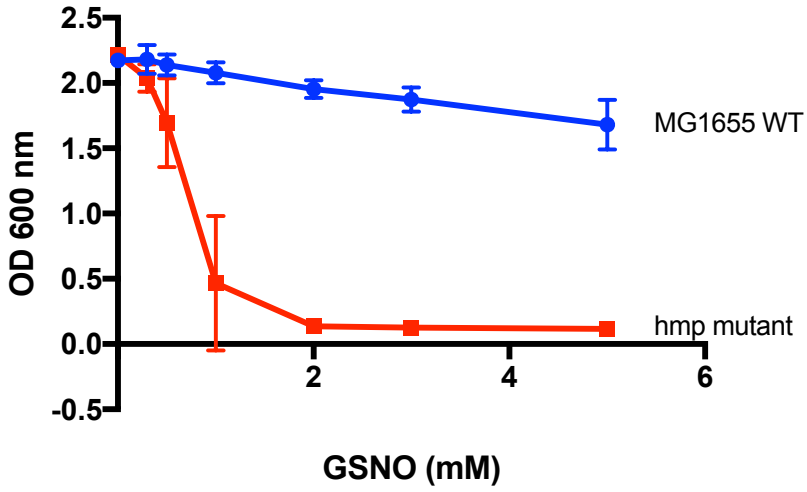


Figure 3.1 The effect of various concentrations of GSNO on the growth of the *hmp* mutant and wild type strains.

The *hmp* mutant and wild type strains were grown in Evans medium. Evans medium (2 ml) was inoculated with 5% (v/v) of overnight starter then supplemented with different concentrations of GSNO (0, 0.3, 0.5, 1, 3, 5 mM). Cultures were grown overnight at 37 ºC, 200 rpm and OD_{600} was recorded. Control experiments were carried out in the absence of GSNO. Data presented are the mean of 3 biological repeats ± S.E.

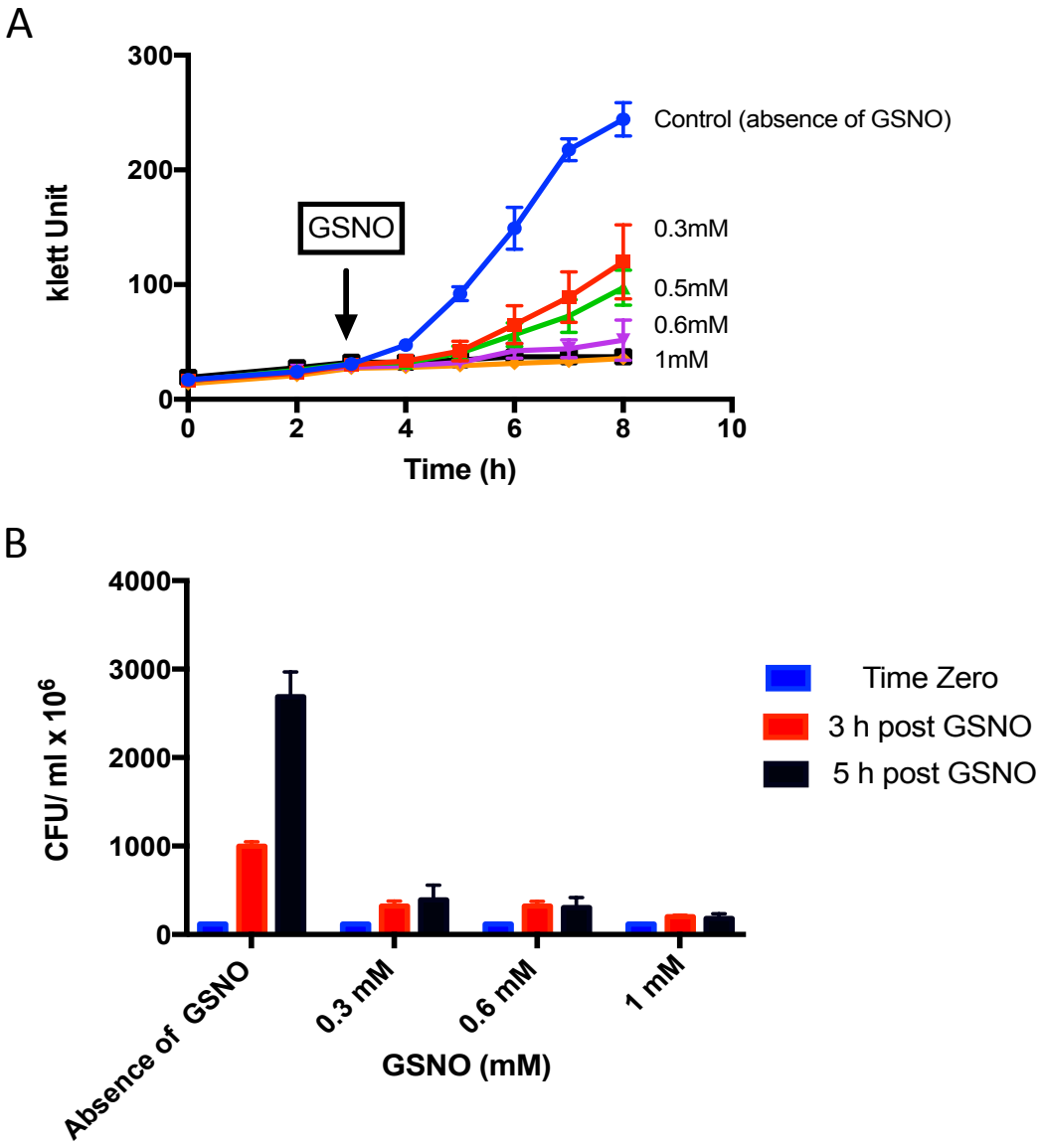


Figure 3.2 The effect of various concentrations of GSNO on the growth and viability of the *hmp* mutant strain

(A) The *hmp* strain was grown in 30 ml Evans medium. GSNO was added at different concentrations (0, 0.3, 0.5, 0.6,0.8 and 1 mM) at 30 Klett units. The OD was recorded at 600 nm every h. (C) The viable count was determined at three different time points: time 0 was the time before adding the GSNO; time 3 was 3 h after adding GSNO; and time 5 was 5 h after adding the GSNO. Control experiments were carried out in the absence of GSNO. Data presented are the mean of 3 biological repeats ± S.E.

After 5 h treatment, 0.3 mM GSNO caused approximately >80 % reduction in cells viability compared to control and 0.6 mM GSNO decreased approximately 85 % of cells viability. GSNO (1 mM) caused 90 % reduction in cells viability after 3 and 5 h of GSNO treatment compared to untreated cells (Fig 3.2 B). Thus, 1 mM GSNO was enough to prevent the cells growing during the different time points. Cells grew normally during a specific period (0, 3 and 5 h) in the absence of GSNO (Fig 3.2 A and B).

3.2.2 Effects of 1 mM GSNO on growth and recovery of *E. coli*

To study the recovery of the *hmp* mutant strain after exposing these cells to nitrosative stress, a 1 mM concentration of GSNO was used because previous results (Fig 3.2 B) had shown that this concentration of GSNO can stop the growth of the cells. To check that result, 30 ml Evans media supplemented with kanamycin and inoculated with 5% of overnight starter (*hmp* strain) was used. The growth was recorded every h at 600 nm using a spectrophotometer. Once the OD had reached 0.4, the first samples were taken and a viable count was preformed at time 0 before adding the stress (GSNO). Following this, 1 mM GSNO was added to the medium and incubated at 37ºC for 2 h. After the incubation period, the second samples were taken for viable counts. In this experiment, the cells were exposed to nitrosative stress (GSNO) for 2 h (Figure 3.3.). As can be seen in Figure 3.3 A, the growth of the *hmp* mutant exposed to 1 mM GSNO was reduced during the 2 h incubation compared to the control. The viable count at zero time at which OD_{600} = 0.4, gave approximately the same number of cells for both the control and 1 mM GSNO stress (Fig 3.3 B), as suggested by the growth data (3.3 A). After exposing the cells to GSNO (1 mM), the recovery of these cells was studied. The *hmp* mutant was exposed to 1 mM GSNO and incubated for 2 h to study the stress phase. The cells were then centrifuged (5000 rpm for 15 min) then resuspended in 5 ml of Evans medium (Fig 3.4). Evans medium was inoculated with stressed cells and the OD at 600 nm was recorded every h. The viable count was taken at time 0, which was the time when new fresh media was inoculated with stressed cells, and at time 2,which was after 2 h incubation at 37º C, 200 rpm (Fig 3.3).

The data in Figure 3.3 shows that the growth of bacteria had increased by 2 h of incubation. The viable count of cells under stress was slightly increased with 1 mM

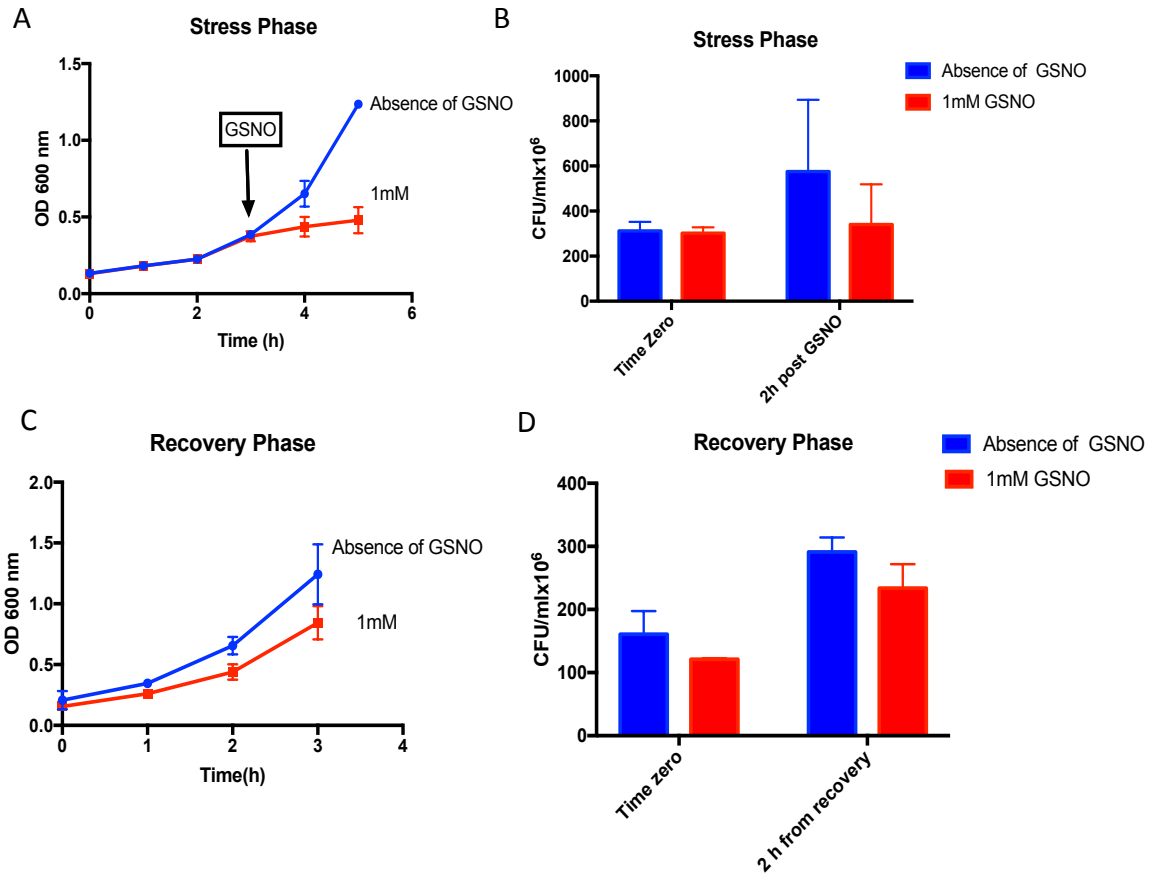


Figure 3.3.The effects of 1 mM GSNO on the growth and recovery of *hmp* mutant strain

The cells were grown in 30 ml Evans Medium supplemented with kanamycin (A) 1 mM GSNO was added at OD_{600}= 0.4 and OD was recorded at 600 nm every h (B) The viable count was taken before adding GSNO, and after 2 h after the addition of GSNO. (C) The cells were harvested and resuspended in 5 ml of Evans media. The fresh medium was inoculated (Recovery Phase) and the OD_{600} was recorded every h. (D) The viable count was taken after washing the cells, which was done at time 0 and 2 h later. Control experiments were carried out in the absence of GSNO. Data presented are the mean of 3 biological repeats ± S.E.

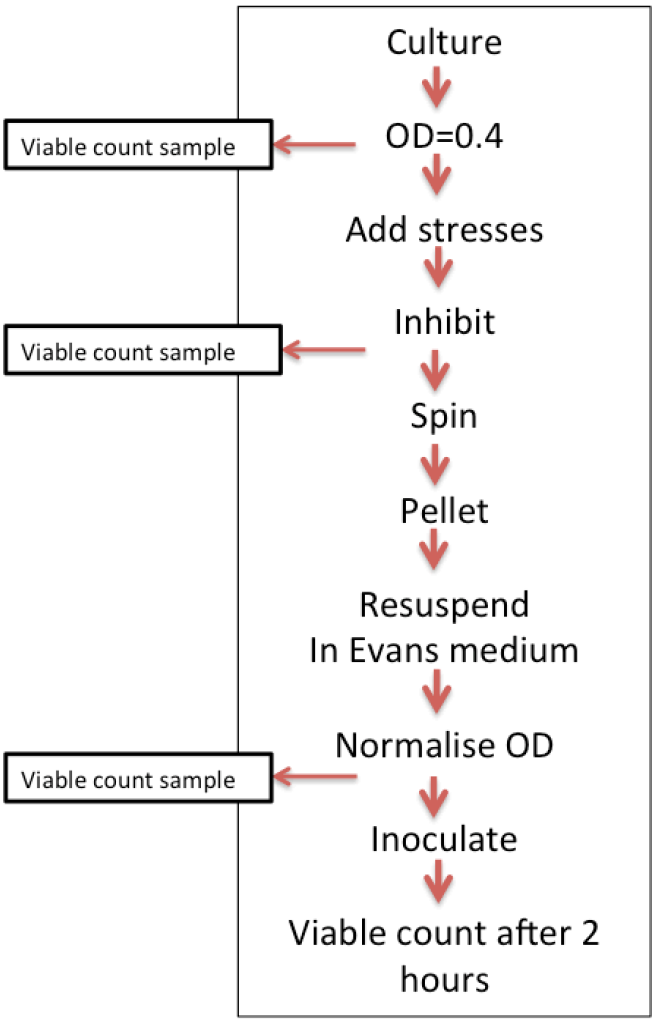


Figure 3.4 Flow chart describing the recovery experiment

of GSNO compared to the time zero (before adding GSNO) (Fig 3.3 D); therefore, these conditions have not achieved 90 % killing and so are not ideal to allow us to study the recovery of *E. coli* from intense stress.

3.2.3 GSNO has a bacteriostatic effect on *E. coli hmp* mutant strain

In order to assess the ability of GSNO to kill 90% of bacterial cells, toxicity of GSNO was tested to identify whether the concentration of cells could affect GSNO toxicity. A similar method has been used to study the toxicity of peroxynitrite on *E. coli* cells, and it was found that peroxynitrite toxicity was dependent on cell density (McLean et al., 2010b). The *hmp* mutant strain was grown in 30 ml Evans medium supplemented with kanamycin. The cells were grown until > 30 Klett unit. After this, the cells were pelleted and resuspeded in PBS to a final volume of 10 ml. Cells were then diluted to 1/4, 1/40 and 1/400. Each dilution was stressed with 0, 1, 3 and 5 mM GSNO. Cells were incubated for 2 h. The viable count of the cells was determined at two time points: before adding GSNO, and after 2 h. The viable count assay showed that the number of the cells in each dilution did not give a significant difference between the cells that were stressed with GSNO and the control (no GSNO). Thus, GSNO at 1, 3 and 5 mM concentrations was not bactericidal to *E. coli hmp* mutant strain with either high or low concentrations of the cells (Figure 3.5). Due to GSNO having only a bacteriostatic effect on *hmp* mutant strain, we turned to NO donor compounds to determine conditions for bacterial cell survival following stress.

3.2.4 Growth curves and viability of *hmp* mutant strain were slightly affected by different concentrations of NOC-5 and NOC-7

The half-life of NOC-7 at pH 7 and 37 ºC is 5 min while for NOC-5 it is 25 min under the same conditions giving a slow release of NO. Thus, using these two compounds simultaneously can maintain NO in the culture for 30 min. Previous study has used a mixture of NOC-5 and NOC-7 to study the modulation of *hmp* regulation under NO challenge (Cruz-Ramos et al., 2002).

To use NO donors that can release NO in shorter times, 0.1 mM NOC-7 and 0.1 mM NOC-5 were used in this work. The growth and viability experiments were conducted in the presence and absence of NOC-5 and NOC-7. The *E. coli hmp* mutant strain

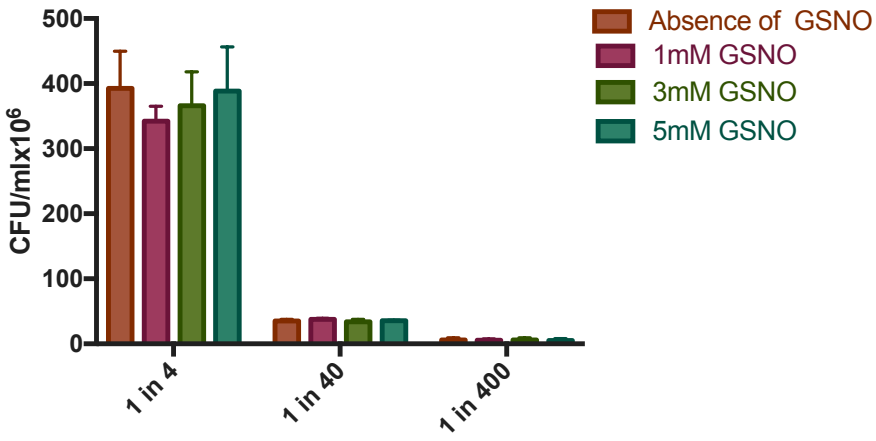


Figure 3.5 The toxicity of GSNO to *E. coli hmp* mutant strain

Cells were grown in 30 ml Evans media until >30 Klett. Cells were pelleted and resuspended in PBS to a final volume of 10 ml. Cells were diluted as above. The dilutions were treated with 1, 3 and 5 mM GSNO. Cells were incubated for 2 h. The viability was determined before adding GSNO, and 2 h post-treatment with GSNO. Data presented are the mean of 3 biological repeats ± S.E.

were grown in 30 ml Evans medium supplemented with kanamycin. NOC-5 and NOC-7 at different concentrations (0, and each at 0.1, 0.3, 0.5, 1 and 3 mM) were simultaneously added at 30 Klett Units (Fig. 3.6 and 3.7). A viable count was taken before adding NOC-5 and NOC-7 and after 30 min and 60 min incubation (Fig 3.6 B and 3.7). The growth curve shows that 0.1 mM of each of NOC-5 and NOC-7 caused inhibition in the growth of the *hmp* mutant strain and 0.3 and 0.5 mM showed a significant reduction in the growth (Fig 3.6 A). The viable count results shows that 0.1 and 0.3 mM of each NOC compounds caused approximately 10 % reduction in cells viability at 30 min while 0.5 mM showed 30 % decrease in viability compared to untreated culture (Fig 3.6 B). After 60 min incubation, 0.1, 0.3 and 0.5 mM of each NOC compounds caused 40 % reduction in viability compared to control (Fig 3.6 B).

Higher concentrations were then evaluated and the viability assay shows that 1 mM of each NOC-5 and NOC-7 caused ~ 35 % reduction in the number of the cells after 30 min incubation compared to control. After 60 min incubation, 1 mM of each NOC compound caused 60 % reduction in cell viability. (Fig 3.7) while 3 mM of NOC-5 and NOC-7 caused 50 % and 70% reduction in cell viability at 30 min and 60 min respectively (Fig 3.7). For low concentrations of NOC-5 and NOC-7, there was no significant inhibition in the growth and viability (Fig 3.6 A and B) while the high concentrations of NOC-5 and NOC-7 showed significant reduction in growth and viability of *hmp* mutant strain up to 70 % after 1 h incubation compared to untreated cells. Thus, these compounds did not achieve the killing of 90% of the bacterial population. However, these compounds are more useful for testing the effect of NO in short period of time not to exceed 60 min.

3.2.5 The viability of the *hmp* mutant and wild type strains exposed to different concentrations of DETA NONOate (NOC-18)

Three reasons make DETA NONOate (NOC-18) the most commonly used NO donor: (i) it can maintain NO levels over a long time period; (ii) the presence of factors such as light, metals, thiol or cells does not change the spontaneous break-down of DETA NONOate as occurs with other NO donors; (iii) its sole decomposition products are NO and non-toxic diethyllenetriamine (Thompson et al., 2009). DETA NONOate has

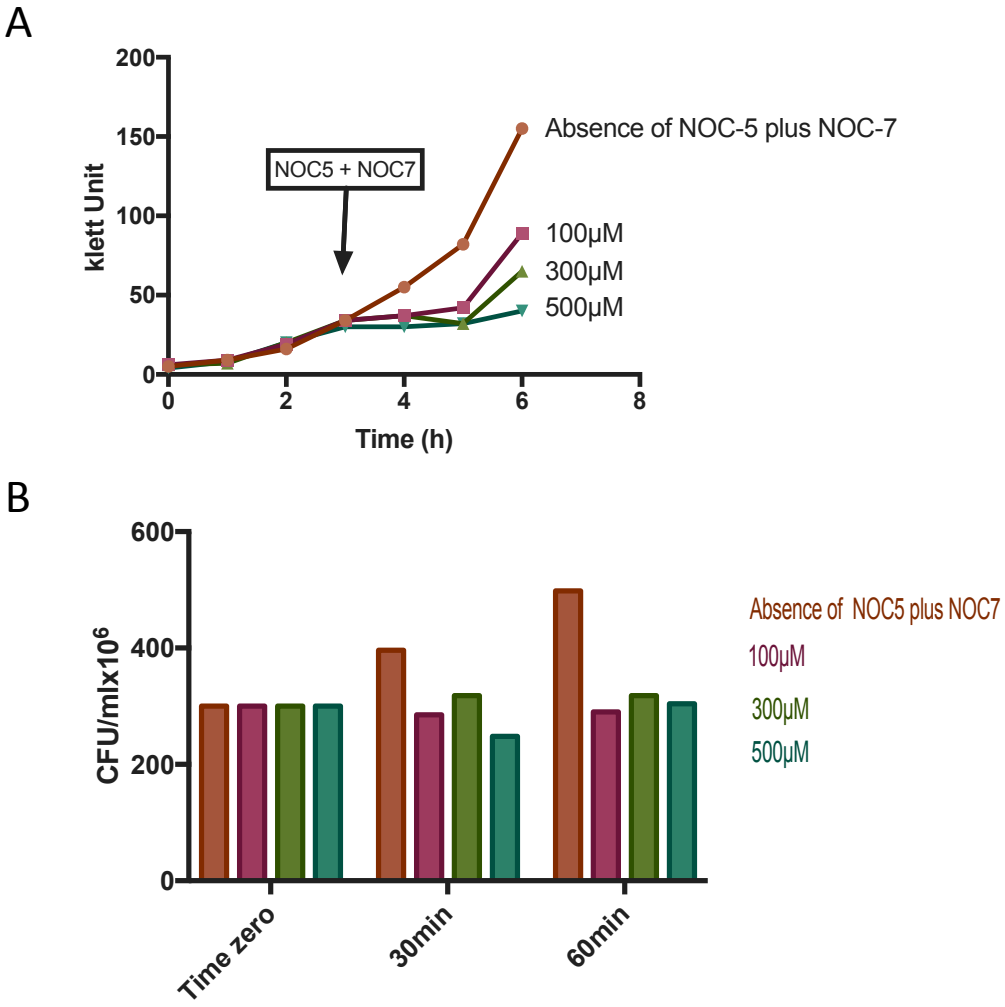


Figure 3.6 Growth curve and viability of the mutant strain were affected by different concentrations of NOC-5 and NOC-7.

Cells were grown in Evans media. (A) At a turbidity of 30 klett unit, NOC-5 and NOC-7 were added in different concentrations (0.1, 0.3 and 0.5 mM). Turbidity was measured every h. (B) The viability was determined at three different time points (0, 30 min and 60 min).

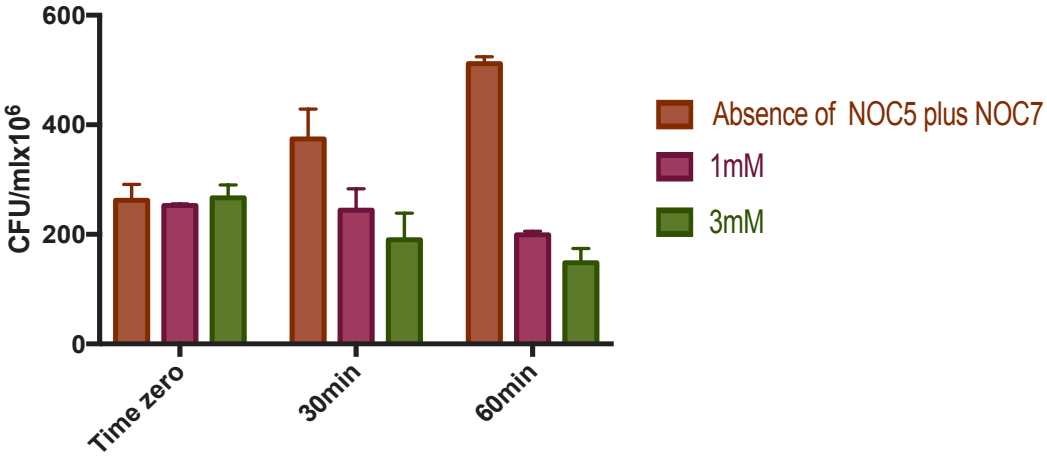


Figure 3.7 Viability of *E. coli hmp* mutant strain were affected by different concentrations of NOC-5 and NOC-7.

Cells were grown in Evans media. At a turbidity of 30 klett unit, NOC-5 and NOC-7 were added in different concentrations (0, 1 and 3 mM). The viability was determined at three different time points (0, 30 min and 60 min). Data presented are the mean of 3 biological repeats ± S.E.

a half-life of 20 h at 37 °C and pH 7.4. It spontaneously liberates 2 equivalents of NO per mole.

The *E. coli hmp* strain was grown in 10 ml Evans medium supplemented with kanamycin and DETA NONOate was used as source of nitrosative stress at different concentrations (0, 0.3, 1 and 3 mM). DETA NONOate was added at 30 Klett unit. The viable count was determined after overnight incubation. The viable count of the *hmp* mutant strain with 1 mM DETA NONOate stress was approximately 50% of the viability of the control while 3 mM DETA NONOate killed about 90% of the cells (Fig 3.8).

In order to study the effects of using deferent growth media on the growth and viability of *E.coli* subjected to DETA NONOate. Flatley et al. (2005) found that Fur-regulated genes did not show any response to GSNO when defined media were utilized to study the expression of these genes. However, these genes were up-regulated when LB medium were used due to the limitation of iron levels in rich medium which these genes need to be derepressed. Thus, they found that the choice of the growth medium can abolish the effect of GSNO on Fur.

Therefore, wild type and *hmp* strains were cultured in Evans medium or LB medium, stressed with addition of 3 mM DETA NONOate at 30 Klett unit and monitored for growth every h (Fig 3.9 A). Viable counts were taken before adding DETA NONOate and after a 5 h and 18 h incubation (Fig 3.9 B). Results demonstrate that there is no significant difference in the growth or viability of wild type and *hmp* strains when these cultures grew in defined minimal medium or LB medium. Moreover, 3 mM DETA NONOate gave a significant reduction of the cell viability in the *hmp* strain while this concentration was not enough to give 90% killing (Fig 3.9 B). These findings demonstrate that the *hmp* strain is more sensitive to the NO.

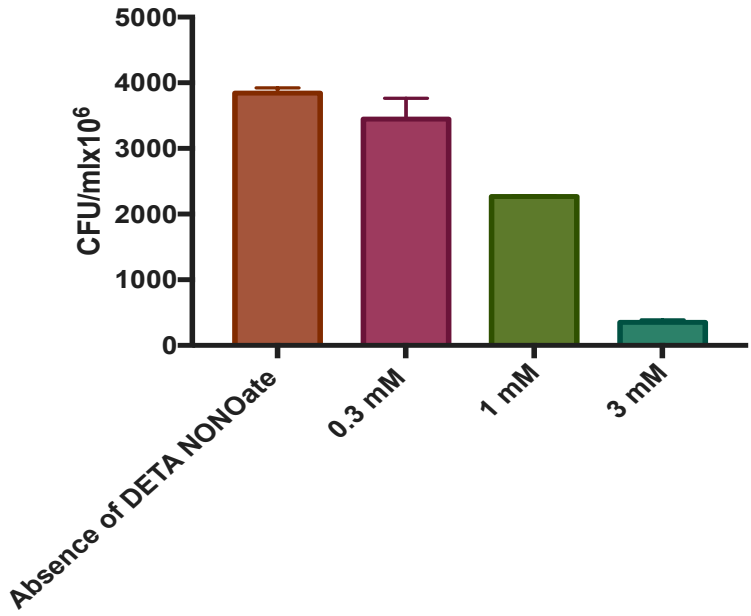


Figure 3.8. The Viable Count of *hmp* mutant strain which was exposed to different concentrations of DETA NONOate (NOC-18)

The *hmp* mutant strain was grown in 10 ml Evans Media. The DETA NONO ate was added in different concentrations (0, 0.3, 1 and 3 mM) at 30 Klett units. Viable Count were performed after overnight incubation. Data presented are the mean of 3 biological repeats ± S.E.

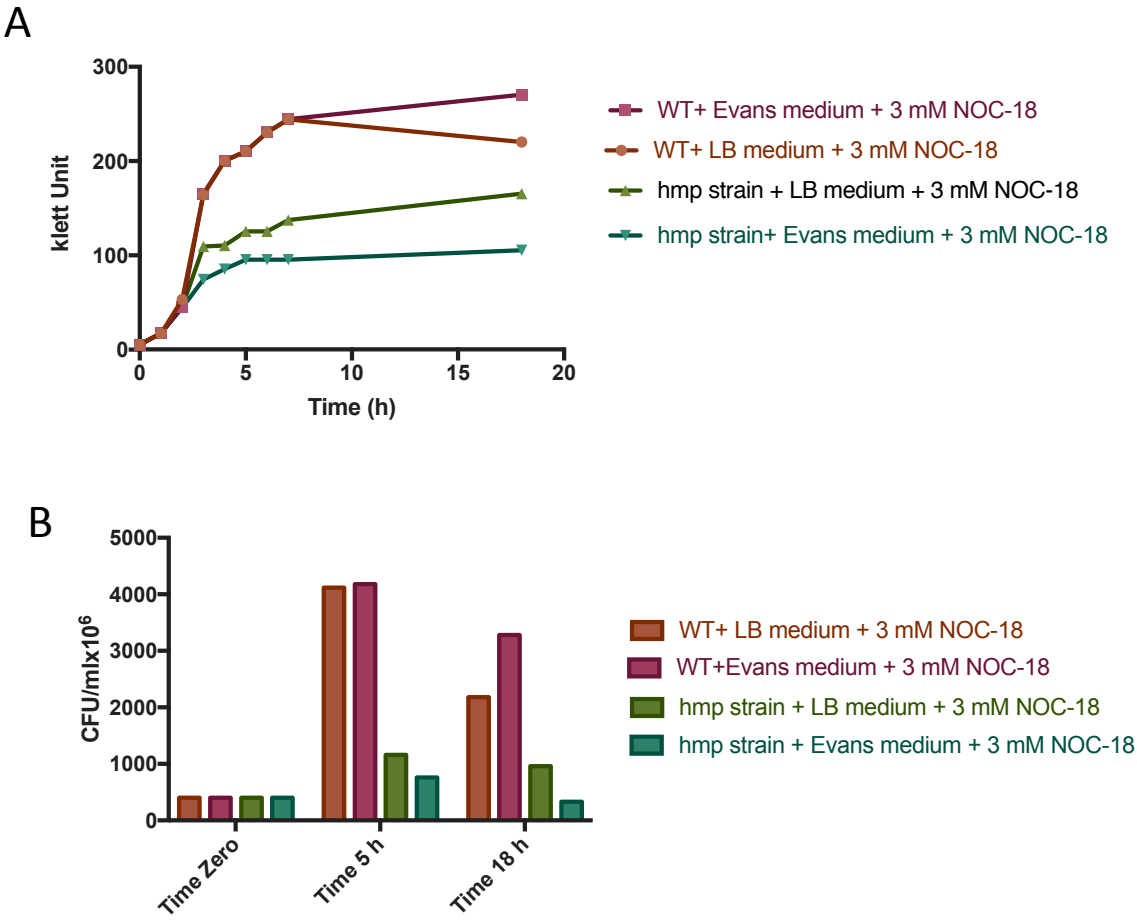


Figure 3.9. Growth curve and viability of wild type and *hmp* mutant exposed to 3 mM DETA NONOate (NOC-18)

The *hmp* mutant strain was grown in 10 ml Evans medium or 10 ml LB medium. Then, 3 mM DETA NONOate (NOC-18) was added at 30 Klett units. (A) Turbidity was measured every h. (B) The viable count was taken with and without 3 mM DETA NONOate (NOC-18) at time points (0, 5 h and 18 h).

3.2.6. Synergistic effect of hydrogen peroxide (H_2O_2)

Previous studies demonstrated that NO alone has bacteriostatic effects on both Gram-negative and Gram-positive bacteria while exposing these cells to nitric oxide combined with hydrogen peroxide increases the bactericidal effects of each compounds (Pacelli et al., 1995). Furthermore, the synergistic effect of nitric oxide with hydrogen peroxide depended on the duration of the stress (Yadav *et al.,* 2014). Nobre and Saraiva (2013) found that combining high concentrations of GSNO with H_2O_2 caused 80 % reduction in *S. aureus* viability and showed a synergistic effect in killing.

The wild type strain was grown in 10 ml Evans medium. Different concentrations of H_2O_2 (0, 2 and 3 mM) and DETA NONOate (0, 1 and 2 mM) were simultaneously and/or individually added at 30 Klett Units (Fig 3.10 A).

Results demonstrated a mild growth inhibitory effect in wild type cells stressed with two compounds (DETA NONOate plus H_2O_2) relative to wild type cells stressed with each compound separately. For the *hmp* strain, cells were grown in the same conditions and different concentrations of H_2O_2 (0, 2, 3 and 5 mM) were added in cobination with DETA NONOate (NOC-18) (0, 1, 2 and 3 mM) at 30 Klett Units. The viable count was determined after overnight incubation (Fig 3.10 B). Figure 3.10 B shows that 2 mM of DETA NONOate with 3 mM H_2O_2 causeed a significant reduction in the number of cells relative to the unstressed controls. The significant reduction in the number of the cells also occurred with low concentrations of theses two compounds (1 mM DETA NONOate plus 2 mM H_2O_2). Moreover, combined stress at high concentrations such as 5 mM of DETA NONOate and 3 mM H_2O_2 inhibited cell viability. In conclusion, addition of 3 mM hydrogen peroxide combined with 2 mM NO inhibited about 90 % of *hmp* strain viability while using the same concentration had a small effect on wild type viability.

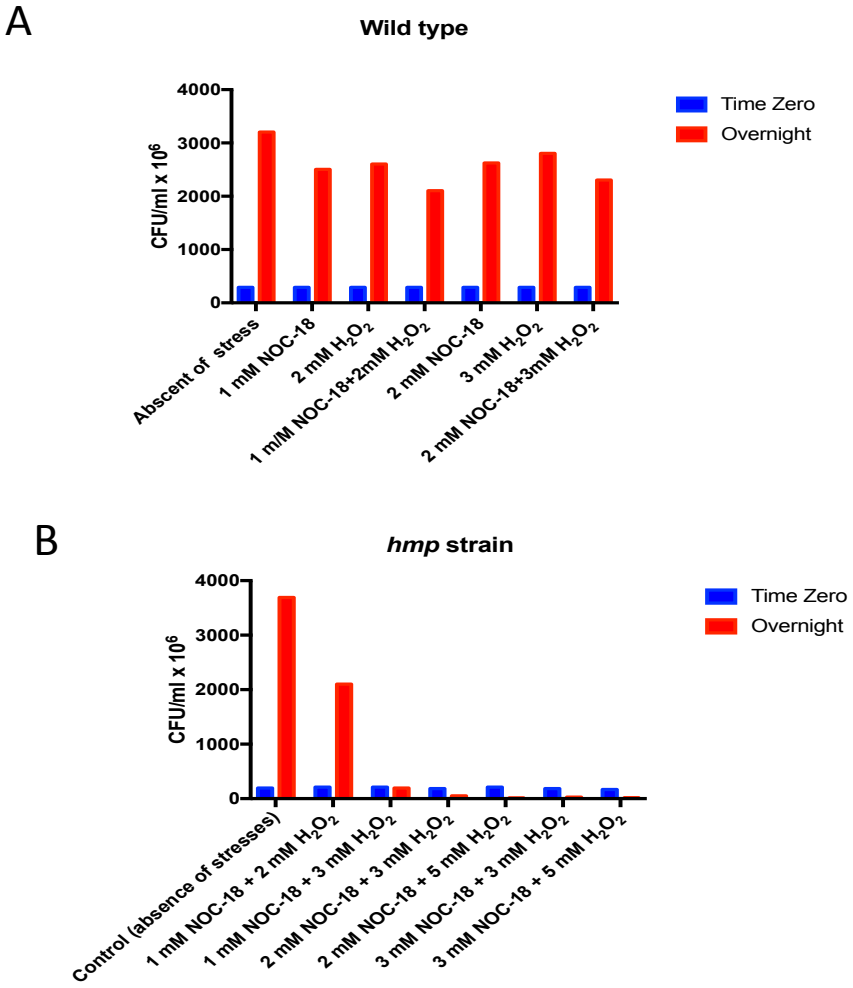


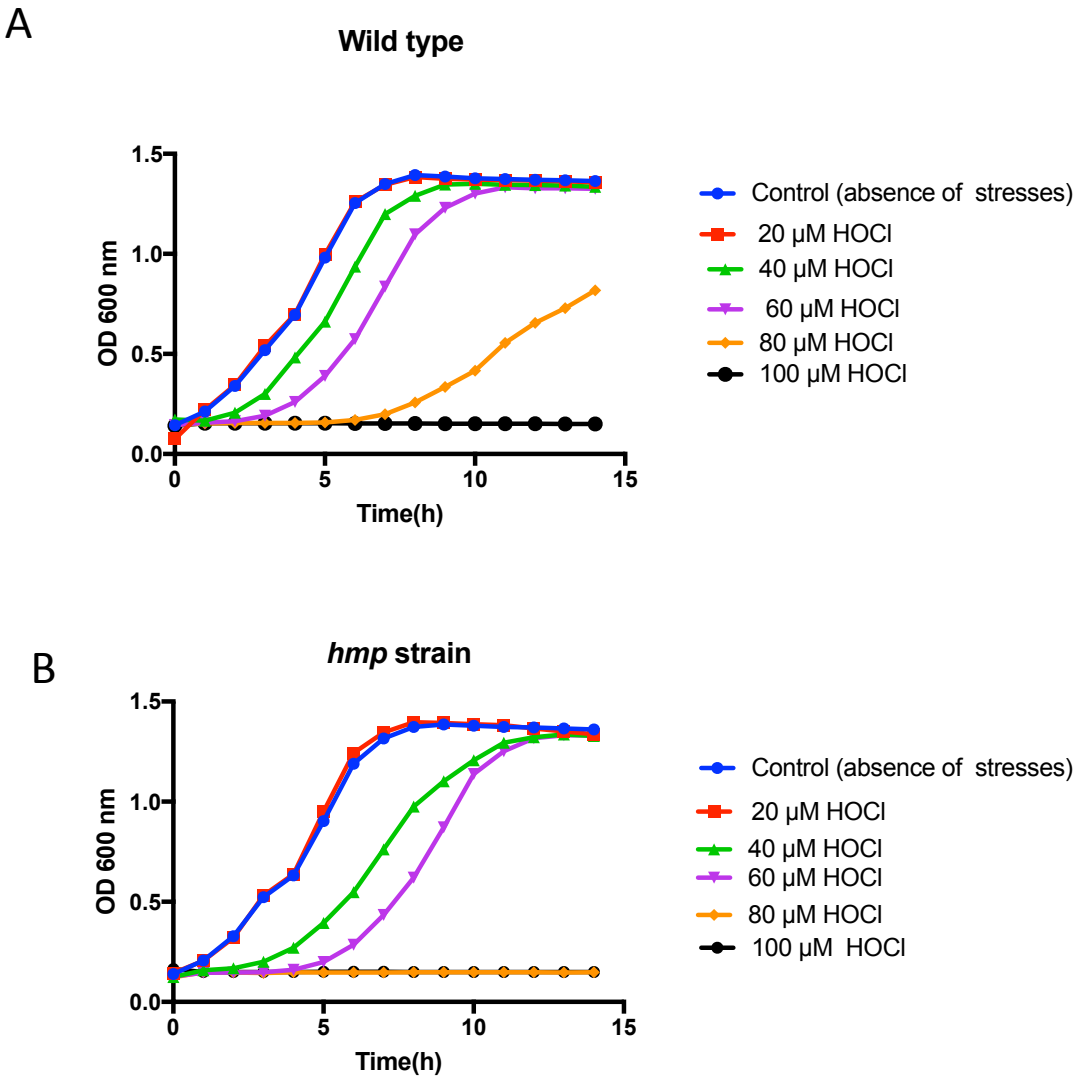
Figure 3.10. The effect of Hydrogen peroxide combined with DETA NONOate (NOC-18)

(A) Wild type strain was grown in Evans medium. Different concentrations of DETA NONOate (NOC-18) (0, 1 and 2 mM) were added individually or simultaneously with H_2O_2 (0, 2 and 3 mM) at 30 Klett. (B) The *hmp* strain was grown in Evans medium. Different concentrations of DETA NONOate (NOC-18) (0, 1, 2 and 3 mM) were added in combination with H_2O_2 (0, 2, 3 and 5 mM) at 30 klett. Cultures were grown at 37 ºC, 200 rpm. The viable count was taken with and without the stresses at zero time before adding the stresses (blue bars) and after overnight incubation (red bars). Control experiments were carried out in the absence of stress.

3.2.7. DETA NONOate, H_2O_2 and HOCl inhibit the growth of both *E. coli hmp* mutant and wild type strains

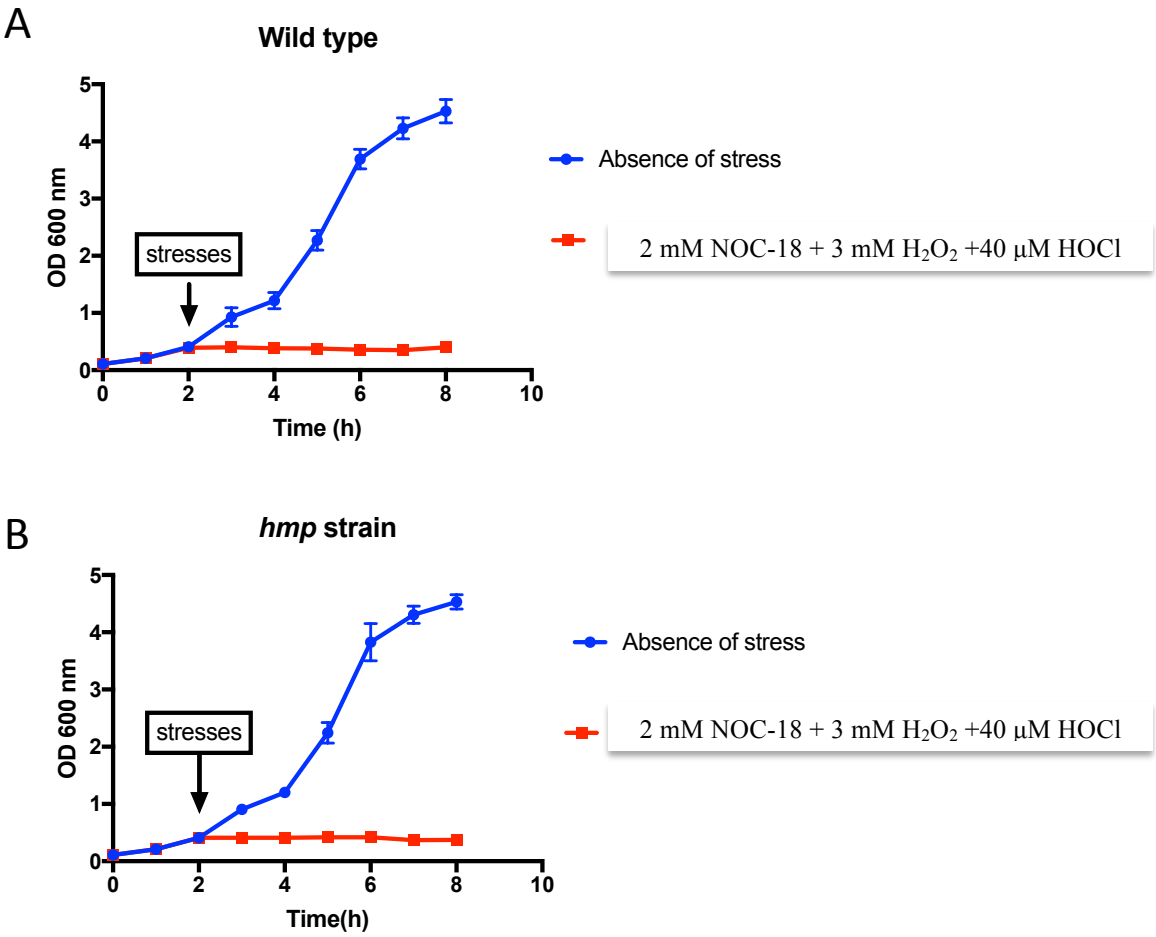
In phagocytes, superoxide and hydrogen peroxide have minor roles in pathogen killing. Therefore, phagocytes need high concentrations and prolonged incubation times of these reactive oxygen species to have bactericidal effect on pathogens. Therefore, phagocytic cells also produce high amounts of other oxidants such as hypochlorous acid (HOCl) to eliminate internalized pathogen (reviewed in (Gray et al., 2013a)).

In order to have 90% killing of bacterial cells in the present work HOCl was added to the other compounds to enhance the bactericidal effect of oxidative and nitrosative stresses. Various concentrations of HOCl were used to investigate the best concentration of HOCl that caused a significant reduction of bacterial growth together with DETA NONOate and H_2O_2 additions. Wild type and *hmp* strains were grown in 10 ml Evans medium and HOCl was added at different concentrations (0, 20, 40, 60, 80 and 100 μM) (Fig 3.11 A and B). The result established that there was no effect on growth when the cells were treated with 20 μM HOCl in both strains while 40 μM HOCl showed only a mild reduction on the growth of both wild type and mutant strains (Fig 3.11 A and B). HOCl at 80 μM concentration showed a significant reduction in wild type growth while HOCl (100 μM) was enough to stop the growth of wild type after incubation for 14 h (Fig 3.11 A). On the other hand, treating the *hmp* strain with 80 μM HOCl was enough to stop the growth. Based upon these finding, 40 μM of HOCl was selected for combination with DETA NONOate and H_2O_2. Wild type and *hmp* strains were grown in 10 ml Evans medium and DETA NONOate (2 mM) was used to induce nitrosative stress. Hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) were used to induce oxidative stress at 3 and 0.04 mM respectively. The optical density was recorded at 600 nm to demonstrate the effect of DETA NONOate, H_2O_2 and HOCl on the growth of both the *hmp* and wild type strains (Fig 3.12 A and B). Both strains showed significant reductions in growth when these cells were exposed to the combination of the three stresses. Based on these results, recovery studies were done to investigate whether *E. coli* cells would survive and recover from these combined nitrostive and oxidative stresses.



3.11 The effect of different concentrations of HOCl on the growth of wild type and *hmp* mutant strains

Cells were grown in 10 ml Evans medium, HOCl were added at the early exponential phase with concentrations (0, 20, 40, 60, 80 and 100 µM). (A) wild type strain and (B) *hmp* strain. Cultures were grown for 14 h at 37 ºC, 200 rpm and OD_{600} was recorded every h. Control experiments were carried out in the absence of HOCl.



3.12 The effect of 2 mM DETA NONOate, 3 mM of H_2O_2 and 40 µM of HOCl on the growth of *E. coli*

Wild type strain (A) and *hmp* strain (B) Cells were grown in 10 ml Evans medium and inoculated to 5%. All stresses were added at OD_{600} = 0.4 (red line). The growth was recorded every h using the spectrophotometer. Control experiments were carried out in the absence of any stresses (blue line). Data presented are the mean of 3 biological repeats ± S.E.

3.2.8 *E. coli hmp* mutant and wild type strains recovery from DETA NONOate, H_2O_2 and HOCl stresses

In order to study the mechanism of bacterial recovery from intense stress induced by nitrosative and oxidative stresses, high concentrations of DETA NONOate, H_2O_2 and HOCl were used to stress bacterial cells and kill approximately 90% of these cells (Fig. 3.13 B and 3.14 B). To study the recovery of *hmp* mutant and wild type strains after exposing these cells to nitrosative and oxidative stress, 2 mM DETA NONOate, 3 mM of H_2O_2 and 40 μM of HOCl were used as previous results had shown that these concentrations can stop the growth of the cells (Fig 3.12 A and B). The stress and the recovery experiment were conducted as described in a flow chart (Fig 3.4). 10 ml Evans medium supplemented with kanamycin for *hmp* mutant strain and inoculated with 5% (v/v) of overnight starter (Fig 3.14) and wild type strain (Fig 3.13) were used.

The growth was recorded every h at 600 nm using a spectrophotometer. Once the OD had reached 0.4, the first samples and viable count were taken at time 0 before adding the stresses. Following this, 2 mM DETA NONOate, 3 mM H_2O_2 and 40 μM HOCl were simultaneously added to the medium and incubated at 37^{o}C for 2 h. After the incubation period, the second samples were taken for viable counts. In this experiment, the cells were exposed to nitrosative stress and oxidative stress for 6 h (Fig 3.13 A and B)(Fig 3.14 A and B). The growth curve and the viability experiments were undertaken in the presence and the absence of the three stresses.

As can be seen in Fig 3.13 and 3.14 A and B, the control grew as expected; however, the growth of both *hmp* and wild type strains exposed to stresses were inhibited during the 6 h incubation. The viable count at zero time at which OD_{600}= 0.4, gave approximately the same number of cells for both the control and stressed cells. The stressed cells had significant reduction in viability 2, 4, and 6 h compared to the control which contained non-stressed cells.

After exposing the cells to stresses, the recovery of these cells was studied (Fig. 3.13 and 3.14 C and D). After 6 h exposing to the 3 stresses, the cells were centrifuged (5000 for 15 min) then resuspended in 5 ml of Evans medium. Evans medium (10 ml) was inoculated with stressed cells (Fig. 3.13 and 3.14 C and D). The growth curve

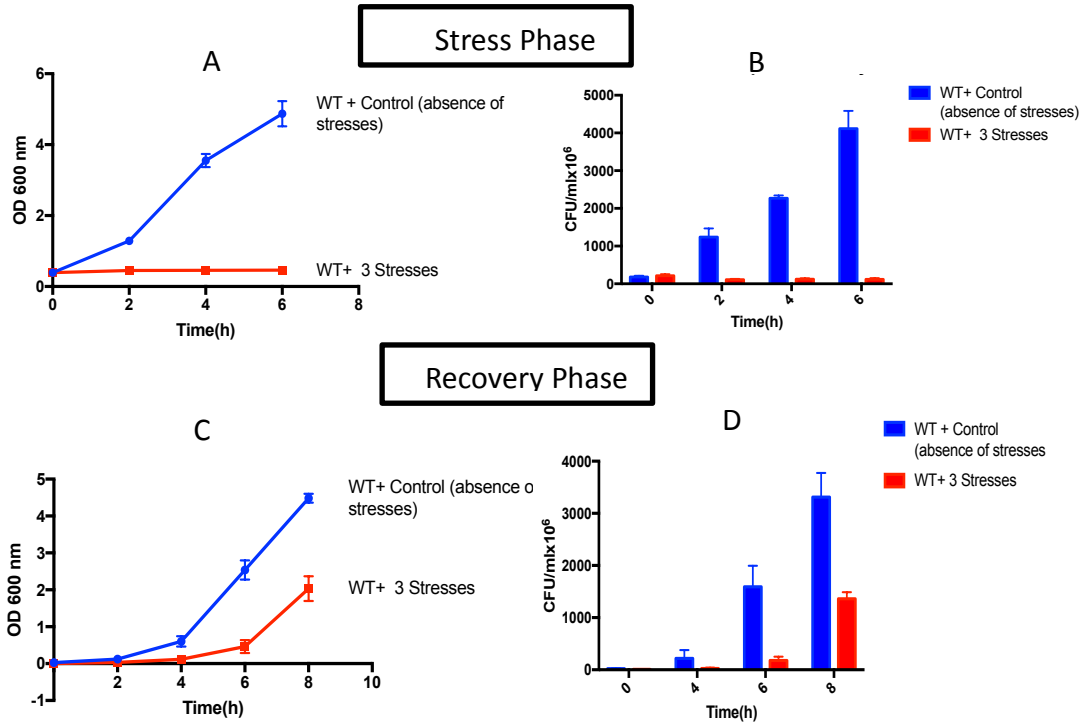


Figure 3.13 The effects of DETA NONOate, H2O2 and HOCl on the growth and the recovery of MG1655.

The cells were grown in 10 ml Evans medium. Cultures were grown at 37 ºC, 200 rpm. (A) DETA NONOate (2 mM), 3 mM H_2O_2 and 40 µM of HOCl were added at OD_{600} = 0.4 as stress phase (red line). The OD was recorded at 600 nm every h using the spectrophotometer. (B) The viable count was taken before adding the stresses, and after 2, 4 and 6 h (red bars) (C) Stressed cells were washed and inoculated to new fresh medium as recovery phase (blue line). The optical density was recorded at 600 nm every h using the spectrophotometer. (D) The viable count was taken every 2 h. Control experiments were carried out in the absence of any stresses (blue line and blue bars). Data presented are the mean of 3 biological repeats ± S.E.

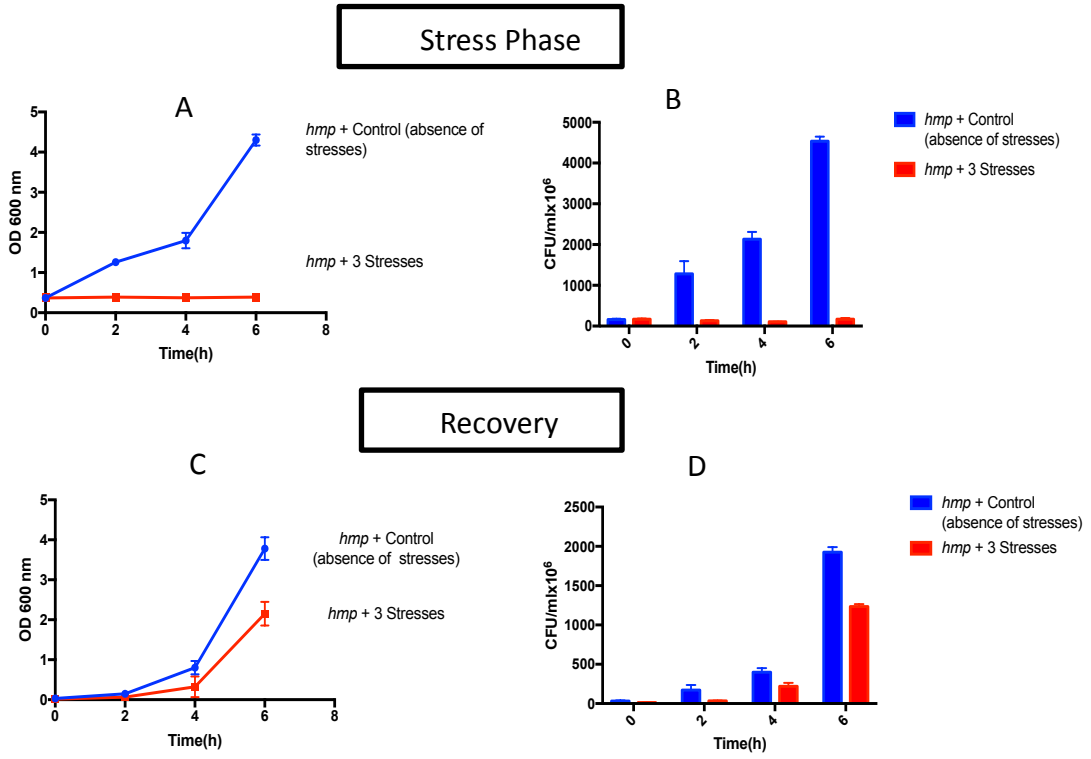


Figure 3.14 The effects of DETA NONOate, H_2O_2 and HOCl on the growth and the recovery of the *hmp* strain

The cells were grown in 10 ml Evans medium. Cultures were grown at 37 ºC, 200 rpm. (A) DETA NONOate (2 mM), 3 mM H_2O_2 and 40 µM of HOCl were added at OD_{600} = 0.4 as stress phase (red line). The OD was recorded at 600 nm every h using the spectrophotometer. (B) The viable count was taken before adding the stresses, and after 2, 4 and 6 h (red bars). (C) Stressed cells were washed and inoculated to new fresh medium in the recovery phase (red line). The optical density was recorded at 600 nm every h using the spectrophotometer. (D) The viable count was taken every 2 h. Control experiments were carried out in the absence of any stresses (blue line and blue bars). Data presented are the mean of 3 biological repeats ± S.E.

was recorded at 600 nm every 2 h (Fig. 3.13 and 3.14 C). The viable count was taken at time 0, which was the time when new fresh media was inoculated with stressed cells, and at 2, 4 and 6 h thereafter (Fig. 3.13 and 3.14 D).

The data in Fig 3.13 and 3.14 C and D show that the growth rate of bacteria had increased by 4-6 h after incubation. The viable count of stressed cells was increased, and the cells recover. These results suggest that both the wild type and *hmp* mutant recovered when the three stresses were removed and the cells transferred to a new fresh medium. *E coli* can induce many different proteins involved in detoxifying and repairing damage caused by nitrosative (Vine and Cole, 2011) and oxidative stresses (Nathan and Shiloh, 2000). The *hmp* mutant strain has approximately the same behavior as wild type when cells exposed to stresses and when these stresses are removed. Therefore, there might be other responsive genes which play a role in the defense and recovery from nitrosative and oxidative stresses.

3.2.9 The effects of DETA NONOate, H_2O_2 and of HOCl on *hmp* Φ(*hmp-lacZ*) expression

The *hmp* gene is subject to dramatic up-regulation by NO and related nitrosative stresses (Poole et al., 1996) but is insensitive to oxidative stress, except at high concentrations (Membrillo-Hernandez et al., 1999). Therefore, *hmp* transcription is a useful measure of the effectiveness of cellular NO-detoxifying systems (Bodenmiller and Spiro, 2006, Pullan et al., 2007)

In order to investigate the mechanism of cells recovery when these cells were subjected to intense stresses, studying the expression of *hmp* was the first step to understand whether *hmp* has a role in cells recovery from nitrosative and oxidative stresses. To study the induction of *hmp* expression after exposing bacteria to nitrosative stress, the *hmp* Φ(*hmp-lacZ*) strain was constructed in this work. This mutant carrying the *hmp* mutation and the Φ(*hmp-lacZ*) fusion were grown in 20 ml Evans medium supplemented with kanamycin for the *hmp* and *hmp* Φ(*hmp-lacZ*) and inoculated with 5% of overnight starter. DETA NONOate (100 μM) was added to the culture. The growth was recorded every h at 600 nm using a spectrophotometer. Once

the OD had reached 0.5, the cells were centrifuged then resuspended in working buffer and the OD_{600} was measured again. ß-galactosidase assay was done using the protocol in (Poole *et al.,* 1996). As can be seen in Fig 3.15 A, the presence of 100 µM DETA NONOate cause a 10-fold elevation in the ß-galactosidase activity from the *hmp* mutant strain *hmp-lacZ* fusion. However, as expected no activity was detected in the strain lacking the fusion. Interestingly, the hmp^+strain containing the *hmp-lacZ* fusion showed only an 8-fold increase. This might be explained by the fact that the Hmp is consuming the NO and consequently there is less NO available for induction of the fusion.

To determine the effects of 2 mM DETA NONOate, 3 mM H_2O_2 and 40 µM HOCl on the *hmp* Φ(*hmp-lacZ*) expression, cells were grown in 20 ml Evans medium supplemented with kanamycin and inoculated with 5% of overnight starter.The growth was recorded every h at 600 nm using a spectrophotometer. A mixture of 2 mM DETA NONOate, 3 mM H_2O_2 and 40 µM HOCl were simultaneously added to the growth at OD_{600} = 0.4. The cells were incubated at 37 ^oC for 2 h as first sample for the ß-galactosidase assay and for 4 h as the second sample for the assay. Fig 3.15 B shows that the expression of *hmp* Φ(*hmp-lacZ*) was increased approximately 7-fold compared to the control when the cells were exposed to 2 mM DETA NONOate for 2 h and was elevated 8-fold than the control when its exposed to DETA NONOate for 4 h. On the other hand, there was no detectable expression of *hmp* Φ(*hmp-lacZ*) under this condition of the three stresses. A likely explanation is that referring to Fig. 3.13 B, these cells were redacted non-viable by the combined stress over 2 h and no regulated gene expression occurred. An alternative explanation for this result is that the stresses directly inhibited the activity of ß-galactosidase.

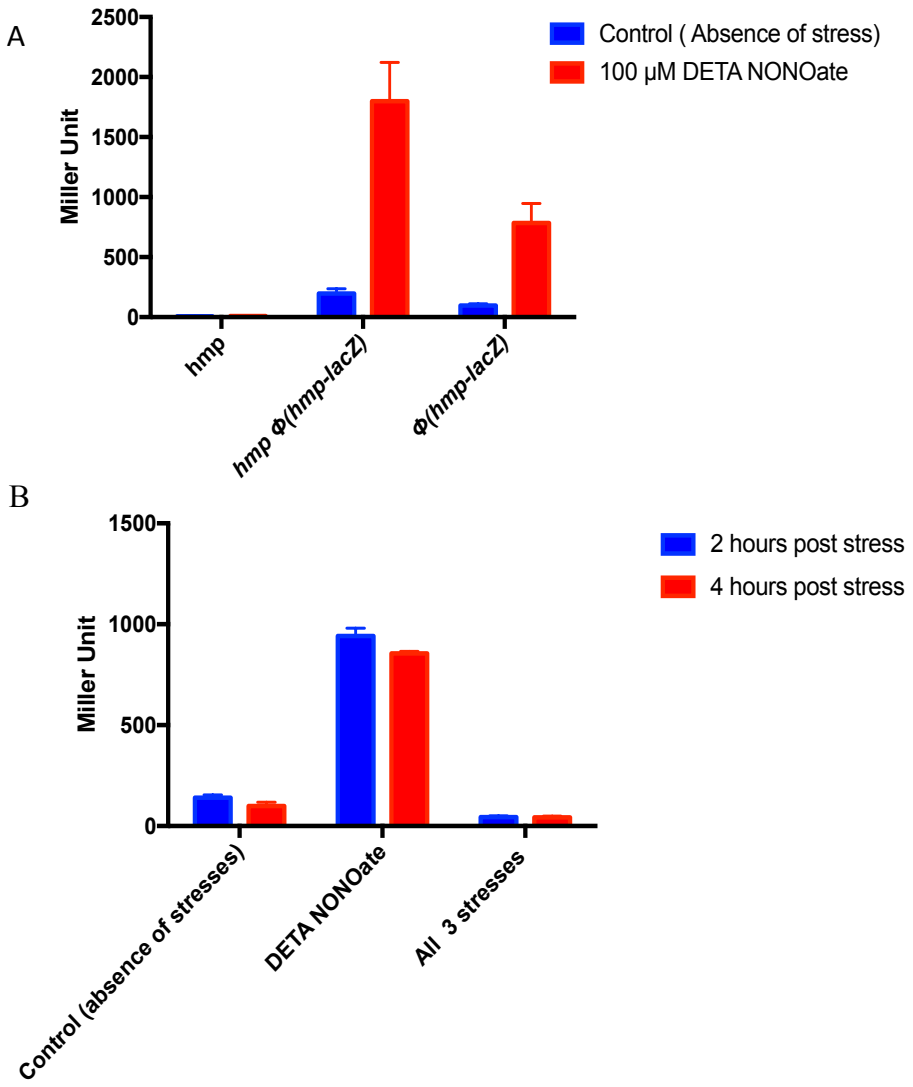


Figure 3.15 The effects of DETA NONOate, H_2O_2 and HOCl on Φ(*hmp-lacZ*) expression in the *hmp* mutant construct

(A) *hmp, hmp* Φ(*hmp-lacZ*) and Φ(*hmp- lacZ*) strains were grown in 20 ml Evans medium supplemented with 100 µM DETA NONOate. The optical density was recorded at 600 nm until OD reach 0.5. The ß-galactosidase assay was conducted to demonstrate the expression of the genes. Control experiments were carried out in the absence of DETA NONOate. (B) *hmp* Φ(*hmp-lacZ*) were grown in 20 ml Evans medium supplemented with kanamycin (Km), 2 mM DETA NONOate, 3 mM of H_2O_2 and 40 µM of HOCl were added at OD_{600} = 0.4. The ß-galactosidase assay was conducted after 2 and 4 h incubation. Control experiments were carried out in the absence of any stresses. Data presented are the mean of 3 biological repeats ± S.E.

3.2.10 The effects of DETA NONOate, H_2O_2 and HOCl on the ß galactosidase enzyme

The results of the previous experiments section appeared to demonstrate low *hmp* Φ(*hmp-lacZ*) expression when cells are exposed to a combination of these stresses. One explanation is that a combination or one of the stresses affects the activity of ß galactosidase. To investigate this, a commercial ß galactosidase (Sigma) enzyme were used to demonstrate whether these reagents could affect the ß galactosidase activity.

ß- galactosidase enzyme (3 µl) was added to 1 ml of working buffer. 2 mM DETA NONOate, 3 mM H_2O_2 and 40 µM HOCl were added to the mixture individually and simultaneously. Then, a 0.2 ml of o-nitrophenyl-β-D –galactoside (ONPG) was added to the mixture. Once the color of the mixture changed to yellow, the optical density was recorded at 420 nm using spectrophotometer. As can be seen in Fig. 3.16, there was no significant difference in the activity of ß galactosidase when exposed to the stresses *in vitro.* From this result, it is likely that these stresses do not affect the activity of ß- galactosidase enzyme but rather gene expression.

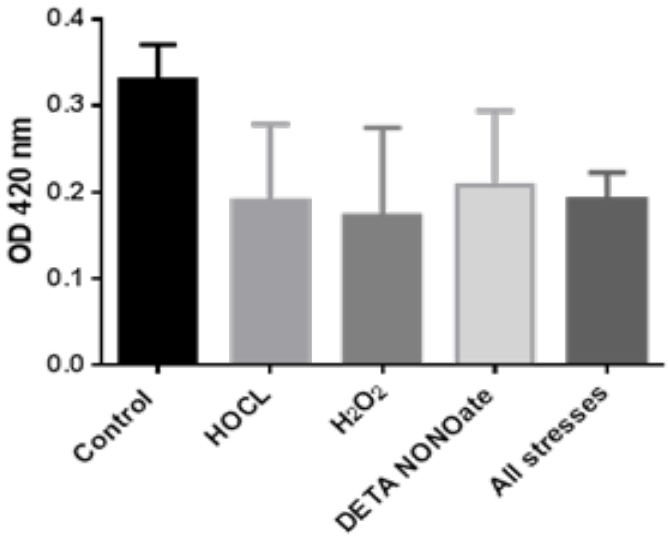


Figure 3.16 The effects of 2 mM DETA NONOate, 3 mM of H_2O_2 and 40 µM of HOCl on the ß galactosidase enzyme

ß galactosidase enzyme (3 µl) of were added with 1 ml of working buffer and 0.2 ml of o-nitrophenyl-β-D–galactoside (ONPG). The optical density was recorded at 420 nm using a spectrophotometer. Control experiments were carried out in the absence of any stresses.

3.3 Discussion

The aim of this work in this chapter was to study the recovery of cells exposed to nitrosative and oxidative stresses. In order to achieve this, many experiments were done to determine conditions for reduction of ~90% from the total viable cell number. Bacterial cells were then exposed to a cocktail of toxic species including ROS and RNS to imitate in part the situation in macrophages during infection. Previous studies revealed that there is a ~90% reduction of *S. Typhimurium* cells within 2 h inside the human macrophage (Stevanin et al., 2002). In this work, *hmp* strain was used alongside wild type as a system to study NO response systems. A similar system has previously been applied in yeast (Horan et al., 2006). Flavohaemoglobin (Hmp) is well established as the main NO detoxification enzyme in many bacteria such as *E. coli* (Forrester and Foster, 2012); there might be other NO responsive genes, which also play a role in the defense against NO and lead to survival of cells from both nitrosative and oxidative stresses.

The data in Fig. 3.2 clearly indicate that the viability of the *hmp* strain had increased when these cells were treated with 1 mM GSNO within 2 h incubation. Therefore, a toxicity experiment was conducted to confirm the toxicity of GSNO on *hmp* strain cell viability (Fig 3.3). GSNO at different concentrations did not have a bactericidal effect on the *hmp* strain cells. Therefore, we found that GSNO affected the growth of *E. coli* cells without killing the cells. 0.5 mM of GSNO is known to have bacteriostatic effect on *Salmonella enterica* cells (Bowman et al., 2011). NO also has a bacteriostatic effect in *E. coli*, and targets respiratory enzymes and biosynthesis pathways of branched-chain amino acids (Hyduke *et al.,* 2007). Based on the bacteriostatic effect of GSNO on *hmp* strain, other NO donor compounds were tested in this work in order to study the recovery of bacterial cells from nitrosative stress.

To study the biology of NO, most scientists prefer to use NO donors. The most commonly used NO donors in biology are the diazeniumdiolates (known as "NONOates") reviewed in (Aga and Hughes, 2008, Bowman et al., 2011). Cruz-Ramos *et al.* (2002) used a cocktail of NO donors NOC-5 (half-life of 25 min at 37 °C) and NOC-7 (half-life of 5 min at 37 °C), in order to maintain NO release for a period of time. In our work, we also utilized a mixture of NO donors (NOC-5 and

NOC-7) to study the effect of these compounds on the growth and viability of the *hmp* mutant (Fig. 3.6 and 3.7). The data in Fig. 3.6 demonstrates that the concentrations of NOC-5 and NOC-7 had only a slight effect on the growth or viability inhibition of *hmp* strain cells. Therefore, a further NO donor was utilized to study the recovery from nitrosative stress. The data presented in Fig 3.8 and 3.9 clearly demonstrate that 3 mM of DETA NONOate had a significant effect on the viability of the *hmp* mutant cells while wild type strain cells are more resistant to that concentration. The finding that *hmp* mutant cells are more sensitive to DETA NONOate than wild type (Fig 3.9) supports the conclusion that this strain is lacking Hmp protein which plays a main role in protecting cells from the toxicity of NO. Previous work found that the *E. coli hmp* mutant strain is more sensitive to killing by NO or other nitrosative stress (Membrillo-Hernandez *et al.,* 1999, Stevanin *et al.,* 2007). Another study shown that *Salmonella serovar Typhimurium hmp* mutants have lower survival from macrophages compared to wild type and that is related to the role that Hmp protein play in cells protection from macrophages stresses (Stevanin *et al.,* 2002). However, the fact that the *hmp* mutant can survive at low level in the macrophages shows that these cells survive from the intense stresses in macrophages and other genes may be involved in the mechanisms of cell survival in macrophages.

Activated macrophages and neutrophils produce NO and H_2O_2 and other reactive oxygen species in order to kill microorganisms (reviewed in Ferrari et al. (2011)). Therefore, hydrogen peroxide and hypochlorous acid were used with DETA NONOate to enhance bacterial killing. Earlier studies found that adding any concentration of NO combined with hydrogen peroxide increases the toxic effect (Pacelli *et al.*, 1995, Yadav *et al*., 2014). The data presented in Fig 3.10 show that using DETA NONOate with hydrogen peroxide effectively enhances the bactericidal effect of the compounds. Reactive oxygen species can work individually or synergistically in order to cause damage to bacterial cells. In *E. coli,* low concentrations of H_2O_2 can cause DNA damage to the cells while lethal concentrations can cause damage to many different cellular targets (Fang, 2004). NO alone can cause inhibition of bacterial respiration and that could be the reason for dormant or persistent existence in some of microorganisms (Fang, 2004). Pacelli et al. (1995) study demonstrated that the increase in toxicity of combined H_2O_2 with NO

in aerobic conditions is related to the formation of $ONOO^-$. Other reason of increase toxicity of the combined oxidative and nitrosative stress is the reaction and damages that occur to the iron- sulfur proteins which lead to increase the Fenton reaction and the presence of $^{\bullet}OH$ (Tortora et al., 2007, Jang and Imlay, 2007).

Both wild type and *hmp* strains indicate significant reduction in growth when these cells are exposed to the DETA NONOate, H_2O_2 and HOCl (Fig 3.12). Here, recovery studies have been conducted to investigate whether *E. coli* cells survive and recover from these nitrosative and oxidative stresses (Fig 3.13 and 3.14). *E coli* can induce many different proteins involved in detoxifying and repairing damage caused by nitrosative and oxidative stresses (Pomposiello and Demple, 2001). Since the *hmp* mutant strain has approximately the same wild type behavior in both the recovery and stress phases, there are likely to be other responsive genes play a role in the defense and recover from nitrosative and oxidative stresses.

Based upon the findings presented in Fig. 3.15 B, it is clear that adding the three stresses to the cells cause no significant effect on the ß galactosidase activity compare to the cells were subjected to DETA NONOate alone. The explanation for this is that bacterial cells showed a significant reduction in cell viability when cells were exposed to the three stresses in combination over 2 h as shown in Fig. 3.14 B and then no regulated gene expression was observed. Therefore, an additional experiment was conducted to confirm whether this result is related to the inhibition of the enzyme activity itself or related to the gene expression (Fig. 3.16). Eriksson et al. (2003) studied the gene expression profile of *S. enterica* during the macrophage infection, and they found that bacteria cells were exposed to peroxide stress and the OxyR response, as evidenced by expression of *trxC* that encodes thioredoxin-2. Notably, *hmp* expression is induced at 8 h, coinciding with NO synthesis by iNOS, as well as *norRVW* responsible for anoxic or microaerobic NO detoxification. In the study they utilized a method in order to isolate bacterial RNA from the infected macrophages. Their method relied on 2 key features (i) a quick stabilization of bacterial RNA to avoid RNA degradation and eukaryotic RNA contamination. (ii) the utilization of detergent to disturb the eukaryotic cells that did not influence the integument bacterial membrane (Eriksson *et al.*, 2003). This work is the first time that these three stresses (NO, H_2O_2 and HOCl) have been used in combination to mimic the onslaught

of stresses experienced by bacteria within the macrophage. The work in this thesis goes on to explore the transcriptional response of some other key genes.

Chapter 4

Transcriptional analysis of genes response to nitrosative and oxidative stress under investigation using real time qPCR

Chapter 4. Transcriptional analysis of genes response to nitrosative and oxidative stress under investigation using real time qPCR

4.1 Introduction

Exposure of cells to stress can cause damage to intercellular functions and may additionally cause killing or adaptation. Stress induced mutagenesis or epigenetic changes can facilitate the adaptation (Gundlach and Winter, 2014).

Pathogenic bacteria such as *Salmonella* require adaptation to the environment inside the phagocytic cells to persist and reproduce. A whole profile of gene expression for pathogenic bacteria was demonstrated for the first time by Eriksson et al. (2003). The transition of pathogenic bacteria from an extracellular environment to the phagocytic cell environment elicits several changes in bacterial gene expression. Eriksson et al. (2003) discovered that approximately 20 % of *S. Typhimurium* coding sequences were changed in expression during murine macrophage infection when a comparison was conducted between the gene expression profiles of intercellular bacteria and gene expression profiles from bacteria grown in culture medium. Microarray data showed that the intracellular bacteria showed remarkable changes which affect 919 genes, 384 of them up-regulated and 535 down regulated. These changes in gene expression occurred 4 h after infection. Further small changes in gene expression were observed between 4 h and 12 h. Between 4 h and 8 h, 50 genes had changed in expression and 30 genes were altered between 8 h and 12 h. These results suggest that the initial changes in the gene expression are required for the intercellular bacteria to grow and survive during macrophage infection. *S. Typhimurium* encounter oxidative stress, which induces response genes such as *soxS, sodB, sodC ycfR, ibpA, ibpB, sbp* and *trxC*. The Eriksson et al. (2003) study revealed that the expression of *hmp* was observed at 8 h after infection. The induction of *hmp* occurs as result of the induction of NO synthesis in macrophages.

The innate defense system inside phagocytic cells activates oxidative stresses as the first line of defense after 1 h following infection (Goldman, 1990). After 4 h following infection, pathogenic bacteria start to replicate (Mills and Finlay, 1998). Around 8 h after the infection, macrophages start to induce the production of nitric oxide (Eriksson et al., 2000).

The study of Baptista et al. (2012) revealed that Hmp protects *E.coli* cells at all points during macrophage infection. While *norV* contributes to bacterial survival and protection from macrophage stresses, this protection is time-dependent, specifically during prolonged incubation in macrophages (longer than 8 h). Stevanin et al. (2002) demonstrated that Hmp plays the main role in detoxifying NO, which leads to protection of *Salmonella Typhimurium* against nitrosative stress inside the macrophage. The same study demonstrated that the *Salmonella hmp* mutant is more sensitive to nitrosative stress but not to peroxynitrite or oxidative stress.

Multidrug-resistant uropathogenic *E. coli* (EC958) carrying mutations in *hmp, norVW, cydAB, ytfE* and *nrfA* were tested by Shepherd et al. (2016) study in order to investigate the ability of these strains to survive from human neutrophils or murine macrophages. The study revealed that *hmp, norVW*, *cydAB* and *ytfE* mutants were more sensitive to neutrophil and macrophage killing relative to wild-type EC958 while a *nrfA* mutations was without affect.

A microarray study showed that the expression of *hmp* and *norV* were significantly up-regulated in response to NOC-5 and NOC-7 in both aerobic and anaerobic conditions and RT-PCR confirmed that *norV* was also significantly up-regulated in response NOC-5 and NOC-7 anaerobically. RT-PCR also confirmed an up-regulation in *norV* expression aerobically (Pullan et al., 2007).

In *E.coli*, the transcription factors OxyR and SoxR activate genes in response to oxidative stress in order to eliminate oxidant damage. The SoxRS regulon activates *sodA* (manganese superoxide dismutase) in response to superoxide while the OxyR regulon include *katG* (catalase-peroxidase) and *ahpCF* (alkyl hydroperoxide reductase) in response to H_2O_2 (Pomposiello and Demple, 2001). Zheng et al. (2001) showed a significant up-regulation of OxyR- regulon genes including *katG* and *ahpC* in response to H_2O_2. The induction of these genes was significantly higher (20-fold) in a wild-type strain than in the *oxyR* mutant strain.

The lack of antioxidant mechanisms in a pathogen can convert a highly virulent pathogen into ROS-sensitive pathogen; it is clear that ROS directly causes damage to microbes (Paiva and Bozza, 2014). Genes related to oxidative stress and regulated by OxyR were strongly up-regulated in *E. coli* ingested by normal neutrophils but not by neutrophils lack of oxidase (Staudinger et al., 2002)

Based on the results of the previous chapter and the observation of the effects of adding hydrogen peroxide, hypochlorous acid and DETA NONOate stress on the expression of *hmp* by using the ß-galactosidase assay, it was deemed interesting to determine how other nitrosative and oxidative stress related genes responded to nitrosative and oxidative stresses when *E.coli* was exposed to these stresses individually or/and simultaneously.

4.2 Results

4.2.1 Effects of DETA NONOate, H_2O_2 and HOCl on the viability of *E. coli* MG1655.

To determine the effect of combining the three stresses on *E. coli*, each compound was first tested alone in order to determine a concentration that elicited only a small growth inhibition. A similar method has been used to study the impact of combining oxygen and nitrogen species on *Staphylococcus aureus* viability in order to study the transcriptional responses to the combined stresses (Nobre and Saraiva, 2013). The study revealed a significant reduction in the viability after 1 h post the exposure to GSNO plus H_2O_2 compared to a single stress.

In this study, *E. coli* MG1655 wild-type were grown in 10 ml Evans medium at 37 °C, 200 rpm. Once the OD reached 0.4, 0.3 mM DETA NONOate, 2 mM H_2O_2 and HOCl (0.1 and 0.2 mM; Fig.4.1) and 0.05 mM HOCl (Fig. 4.2 and 4.3) were added individually or simultaneously. A viable count was taken before adding the stresses and after 10 min and 1 h incubation (Fig 4.1, 4.2 and 4.3).

The data in Fig. 4.1 indicated that 0.3 mM DETA NONOate gave a slight reduction in bacterial viability, compared to an untreated culture, after 10 min and a greater inhibition after 1 h. On the other hand, 2 mM H_2O_2 was without significant effect even after 1 h. Exposing the culture to 0.1 mM HOCl caused a significant reduction of approximately 50 % of the cell number compared to unstressed culture at both 10 min and 1 h. The higher concentration of HOCl (0.2 mM) alone was enough to reduce cell counts to below detectable levels. The combined effects of the three stresses were to reduce viability counts substantially at 10 min and totally after 1 h (Fig. 4.1). Further viability tests were conducted to study the effect of combining each two compounds on *E. coli* viability. Fig. 4.2 shows that a mixture of 0.3 mM NONOate, 2 mM H_2O_2 and 50 μM HOCl reduced the viable count by approx. 60%. Similar results with three simultaneous additions of stress reagents are shown in Fig 4.3, but the data also revealed that adding all three were needed to reduce viable counts by >50% after 1 h.

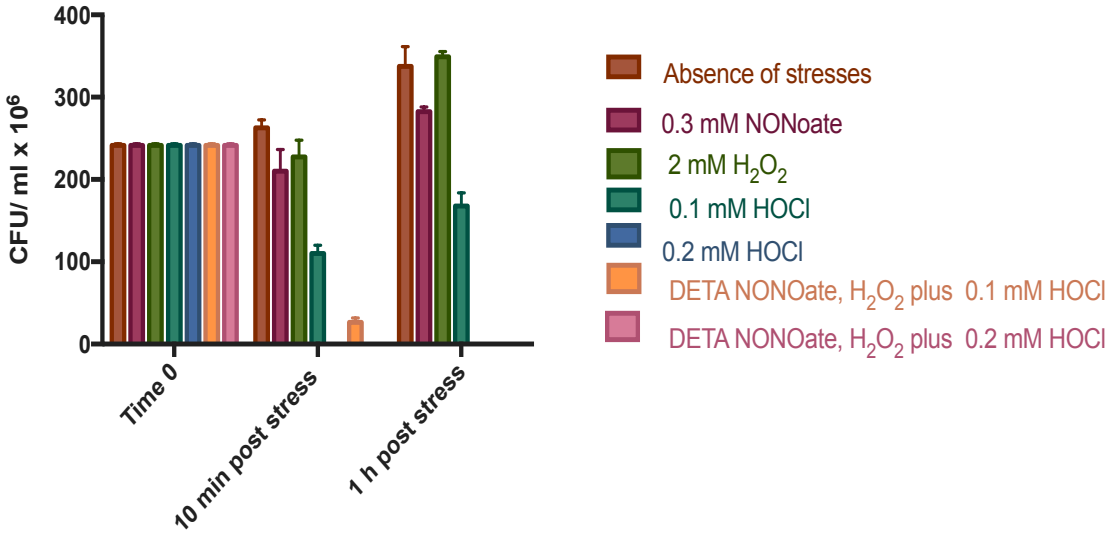


Figure 4.1 The effect of DETA NONOate, H_2O_2 and HOCl on *E. coli* wild-type viability.

MG1655 wild-type were grown in Evans medium. 0.3 mM DETA NONOate, 2 mM H_2O_2 and 0.1 or 0.2 mM of HOCl were added individually or simultaneously at OD_{600}=0.4. The viable count was taken at time zero before adding the stress and 10 min and 1 h post the stress. Data represented are the mean of 3 biological repeats ± S.E.

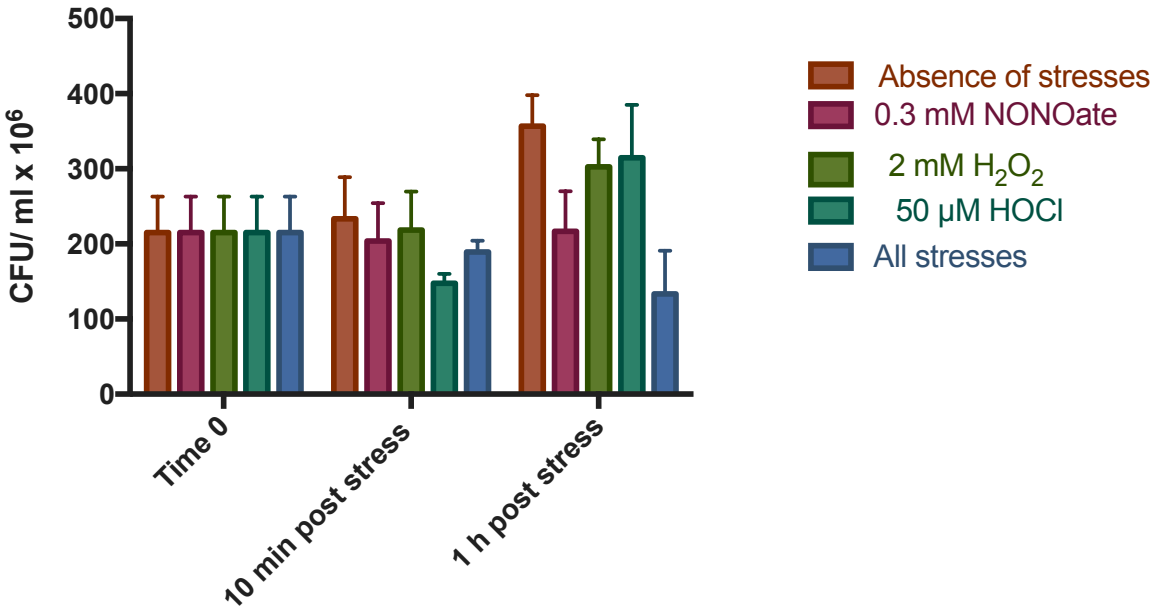


Figure 4.2 The effect of DETA NONOate, H_2O_2 and HOCl on *E. coli* wild-type viability.

MG1655 wild-type were grown in Evans medium. 0.3 mM DETA NONOate, 2 mM H_2O_2 and 50 µM of HOCl were added individually or simultaneously at OD_{600}=0.4. The viable count was taken at time zero before adding the stress and 10 min post stress and 1 h post the stress. Data represented are the mean of 3 biological repeats ± S.E

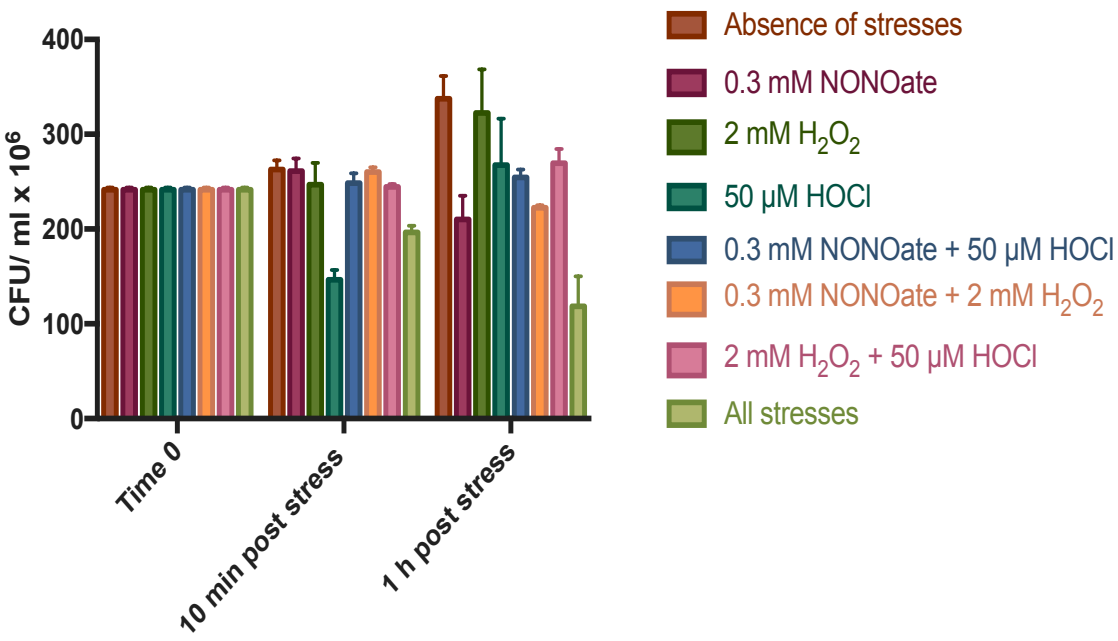


Figure 4.3 The effect of DETA NONOate, H_2O_2 and HOCl on *E. coli* wild type viability.

MG1655 wild type were grown in Evans medium. 0.3 mM DETA NONOate,2 mM H_2O_2 and 50 µM of HOCl were added individually or 0.3 mM DETA NONOate plus 2 mM H_2O_2 or 0.3 mM DETA NONOate plus 50 µM of HOCl or 2 mM H_2O_2 plus 50 µM of HOCl or all three stresses were added simultaneously at OD_{600}=0.4. The viable count was taken at time zero before adding the stress and 10 min post stress and 1 h post the stress. Data represented are the mean of 3 biological repeats ± S.E.

4.2.2 Real Time PCR of oxidative and nitrosative stress genes upon exposure to DETA NONOate, H_2O_2 and HOCl

In order to investigate whether adding DETA NONOate, H_2O_2 and HOCl simultaneously or individually caused transcriptional changes in genes involved in response to oxidative and nitrosative stresses, *E. coli* MG1655 was grown in 10 ml Evans medium. The growth was recorded every hour at 600 nm using a spectrophotometer. Once the OD reached 0.4, DETA NONOate (0.3 mM), H_2O_2 (2 mM) and HOCl (50 μM) were added to the culture for 10 min. Then, a sample was taken for RNA- extraction. RT-PCR analysis of the transcription of *hmp*, *norV*, *katG, ahpC, sodA* and *nemA* was conducted (Fig 4.4).

The *hmp* gene encoding flavohaemoglobin was highly up-regulated 64-fold on treatment with DETA NONOate alone and 64-fold when DETA NONOate was combined with H_2O_2 plus HOCl (Fig. 4.4 A). HOCl or H_2O_2 alone were without effect. Elevation of *hmp* expression in response to NO was expected due to *hmp* encoding the flavohaemoglobin that detoxifies NO (Poole et al., 1996, Gardner et al., 1998). DETA NONOate also elicited up-regulation of *norV* by 294-fold; the flavorubredoxin is also important in detoxifying nitric oxide but primarily in anoxic conditions (Gardner et al., 2002) (Fig. 4.4 B). However, Fig. 4.4 B also showed that the expression of *norV* was up-regulated to a much lesser extent (16-fold) when cells were exposed to DETA NONOate in combination with H_2O_2 and HOCl. This finding may suggest that H_2O_2 might block the transcriptional response of *norV* to DETA NONOate. On the other hand, the *norV* gene was up-regulated ~ 2-fold by H_2O_2 and there was no change due to HOCl (Fig. 4.4 B).

Figure 4.4 C indicates that, as expected, *katG* was up-regulated about 50-fold in response to H_2O_2. When H_2O_2 was combined with DETA NONOate and HOCl, the transcript increase was approximately 70-fold. . However, treating cells with DETA NONOate alone also caused an increase in *katG* expression of approx. 5-fold , while cells exposed to HOCl alone showed a >3 fold up-regulation in *katG* expression. The effects of DETA NONOate and HOCl on *katG* expression are not readily explained. However, peroxynitrite ($ONOO^-$) is formed by the reaction of superoxide with NO.

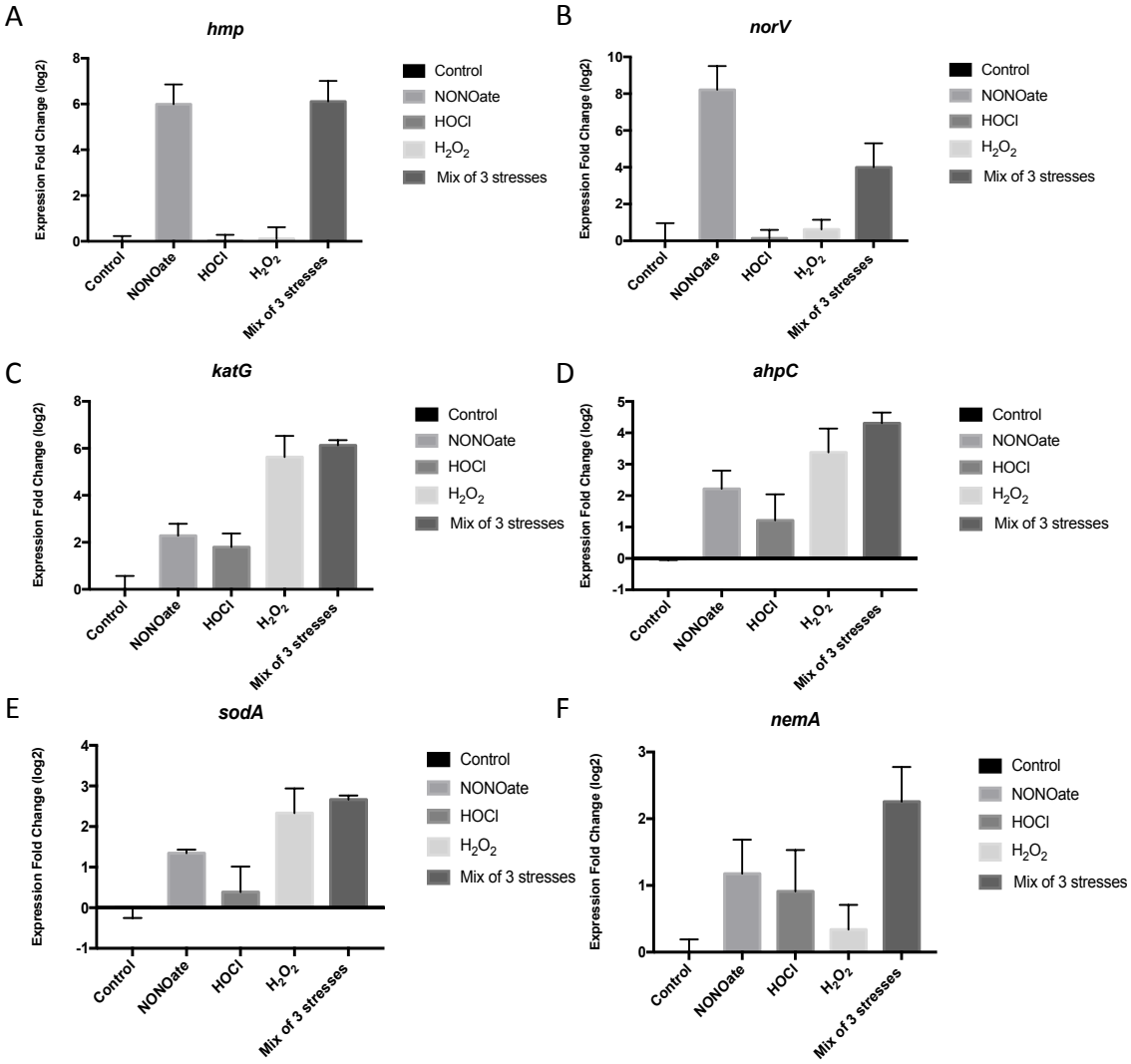


Figure 4.4 Real Time PCR of oxidative and nitrosative stress genes upon exposure to DETA NONOate, H_2O_2 and HOCl

Cultures of *E.coli* MG1655 wild type were treated with 2 mM H_2O_2 0.3 mM DETA NONOate and 0.05 mM HOCl alone or in combination with other compounds at OD_{600}= 0.4 for 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.). (A) *hmp* (B) *norV*. (C) *katG*. (D) *ahpC*. (E) *sodA*. (F) *nemA*.

Microarray and RT-PCR studies on *E. coli* revealed that the *katG* gene was significantly up-regulated in response to peroxynitrite but also by hydrogen peroxide (McLean et al., 2010b). The data may suggest that the combined effect of NONOate and the other stress reagents is to generate peroxynitrite that causes increased expression of *katG*.

Similar results were obtained in studies of *ahpC* (Fig. 4.4D). The a*hpC* gene was up-regulated 10-fold in response to H_2O_2 but more highly expressed (20-fold) in response to the three stresses together, relative to the unstressed control (Fig. 4.4 D). As for *katG*, the individual effects of DETA NONOate (5-fold) and HOCl (>2-fold) on gene expression are not readily explained.

The *sodA* gene encodes superoxide dismutase that degrades this free radical. However, in this work, *sodA* was up-regulated (5-fold) by addition of H_2O_2 and (3-fold) by addition of DETA NONOate. It was also up-regulated (6-fold) by all three stresses combined. No change in gene expression was occurred when cells were exposed to HOCl alone (Fig.4.4 E). The *nemA*, encoding N-ethylmaleimide reductase was up-regulated 5-fold by the three stresses compared to unstressed control, and it However, *nemA* was up-regulated when cells were exposed to nitric oxide and the explanation for this finding is not obvious.

Fig. 4.4 F revealed that the *nemA* gene responded to HOCl as expected from the role that the gene product, N-ethylmaleimide reductase, plays in detoxifying reactive electrophiles produced by HOCl (Hillion and Antelmann, 2015). However, *nemA* was also up-regulated approximately 2-fold in response to DETA NONOate or to HOCl and to the three combined stresses compared to unstressed controls. There was no change in *nemA* expression caused by H_2O_2 (Fig. 4.4 F).

In conclusion, adding the three stresses simultaneously for 10 min causes an increase in oxidative stress-related gene expression compared to unstressed controls (Fig. 4.4).

4.2.3 Real Time PCR of oxidative and nitrosative stress genes upon sequential exposure to DETA NONOate and H_2O_2

In *Salmonella* infection, the initial host response is production of reactive oxygen species that play an essential role in killing pathogenic bacteria, after which reactive nitrogen species play a role in controlling and limiting the replication of pathogenic bacteria. This sequential exposure to ROS and RNS is very important in determining the extent to which *Salmonella* is killed by macrophages (Fang, 2004).

In order to investigate how oxidative and nitrosative related genes were responding to oxidative and/or nitrosative stresses, changes in expression of the genes was monitored following sequential exposure.

In subsequent Figures, where additive and sequential treatments are illustrated, the structure is as follows. Section A shows 10 min exposure to the stressor and B shows 25 min treatment plus the effect of combining the two (i.e. 25 min with each compound). Section C shows first the compound added at time zero (alone), then the second compound (alone) and finally the effect of the sequential additions. Section D shows the inverted order of additions. This presentation necessarily replicates some data bars, since results from one time period of treatment (10 or 25 min) are shown alongside other conditions where two stress compounds are added simultaneously or sequentially (see Figs 4.6 to 4.23).

The cells were grown in 10 ml Evans medium and cultures inoculated with 5 % of overnight starter. The growth was recorded every hour at 600 nm using a spectrophotometer. Once the OD reached 0.4, the first stress or all stresses were added to the culture, then the cells were incubated for 10 min and a sample was taken for RNA-extraction. For sequential exposures, the second stress was added to the culture after 10 min incubation with the first stress. The cells were grown with the second stress for 15 min to give a total of 25 min incubation. Then, a sample was taken for RNA-extraction (Fig 4.5).

Compared to the unstressed control, *hmp* was significantly up-regulated in response to DETA NONOate for 10 min (Fig. 4.6) confirming the findings from a previous result (Fig. 4.4 A) and the known response of *hmp* to NO (Poole et al., 1996). DETA

NONOate triggered a 719-fold up-regulation of *hmp* when cells were exposed to the agent alone for 25 min. Exposing a culture to H_2O_2 (25 min) alone caused no change in *hmp* expression. However, sequential exposure to DETA NONOate first for 10 min then followed by H_2O_2 for 15 min caused an enhanced up-regulation in the expression of *hmp* of ~ 1235 fold (Fig 4.6 C) and the gene was also up-regulated 915-fold when cells were treated with H_2O_2 first followed by DETA NONOate (Fig. 4.6 D). Exposing the cells simultaneously to DETA NONOate plus H_2O_2 caused an increase in the expression of *hmp* by ~ 2019-fold. Thus, *hmp* is very highly expressed in response to DETA NONOate or to DETA NONOate plus H_2O_2 (Fig.4.6).

Fig. 4.7 shows comparable data for a second NO-responsive gene, *norV*. Compared with the unstressed control, the expression of *norV* was ~ 2000-fold up-regulated in response to DETA NONOate in 10 or 25 min (Fig 4.7 A, B) confirming the finding in the previous result (Fig. 4.4B). This result is consistent with the known role of *norV* in NO resistance mechanisms, especially anoxically (Gardner and Gardner, 2002, Gardner et al., 2002). In response to hydrogen peroxide, the expression of *norV* gene was up-regulated approx. >2-fold when cells were exposed to H_2O_2 for 10 min (Fig 4.7 A) while no change in gene expression was observed after 25 min (Fig 4.7 B). Exposure of the cells to DETA NONOate plus H_2O_2 for 25 min caused a smaller up-regulation in the gene expression of approximately 298-fold (Fig 4.7 B). When DETA NONOate was applied to the cells for 10 min followed by treatment with H_2O_2 for a further 15 min, *norV* was up-regulated to a lesser extent (50-fold) (Fig 4.7 C). However, a significant increase in the expression of *norV* (~ 2700-fold) compared to unstressed cells was observed when cells were exposed to H_2O_2 for 10 min then followed with DETA NONOate for 15 min (Fig 4.7 D). This result indicates that treating cells with DETA NONOate first followed with H_2O_2 impairs the expression of *norV*.

As expected, H_2O_2 alone caused up-regulation of *katG* expression (Fig. 4.8A, B). However, the increase was lower after 25 min than at 10 min, suggesting that the response to exogenous peroxide is rapid but short-lived. Surprisingly, DETA NONOate alone also elicited up-regulation of *katG* expression when cells were exposed for either 10 or 25 min by 4-fold and 100-fold respectively (Fig. 4.8 A, B). When cells were exposed to the two stresses simultaneously for 25 min, there was an up-regulation in the expression of *katG* (315-fold) (Fig. 4.8 B).

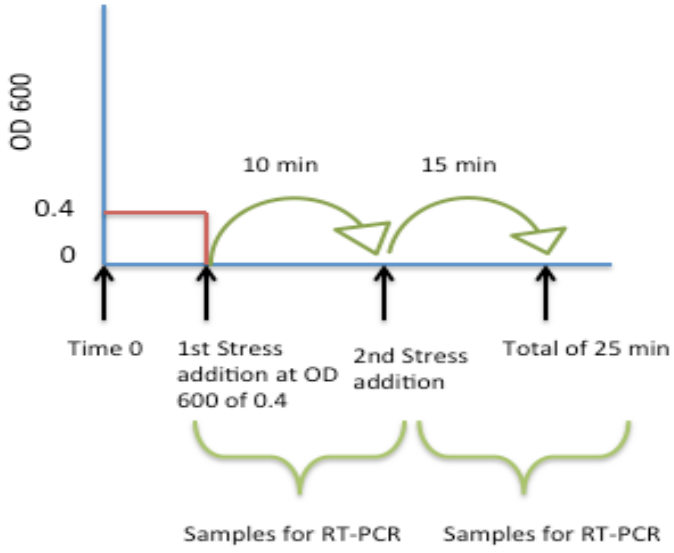


Figure 4.5 Sequential exposures were done by treating the cells to the first stress for 10 min at OD_{600} of 0.4 followed by a 15 min exposure to the second stress.

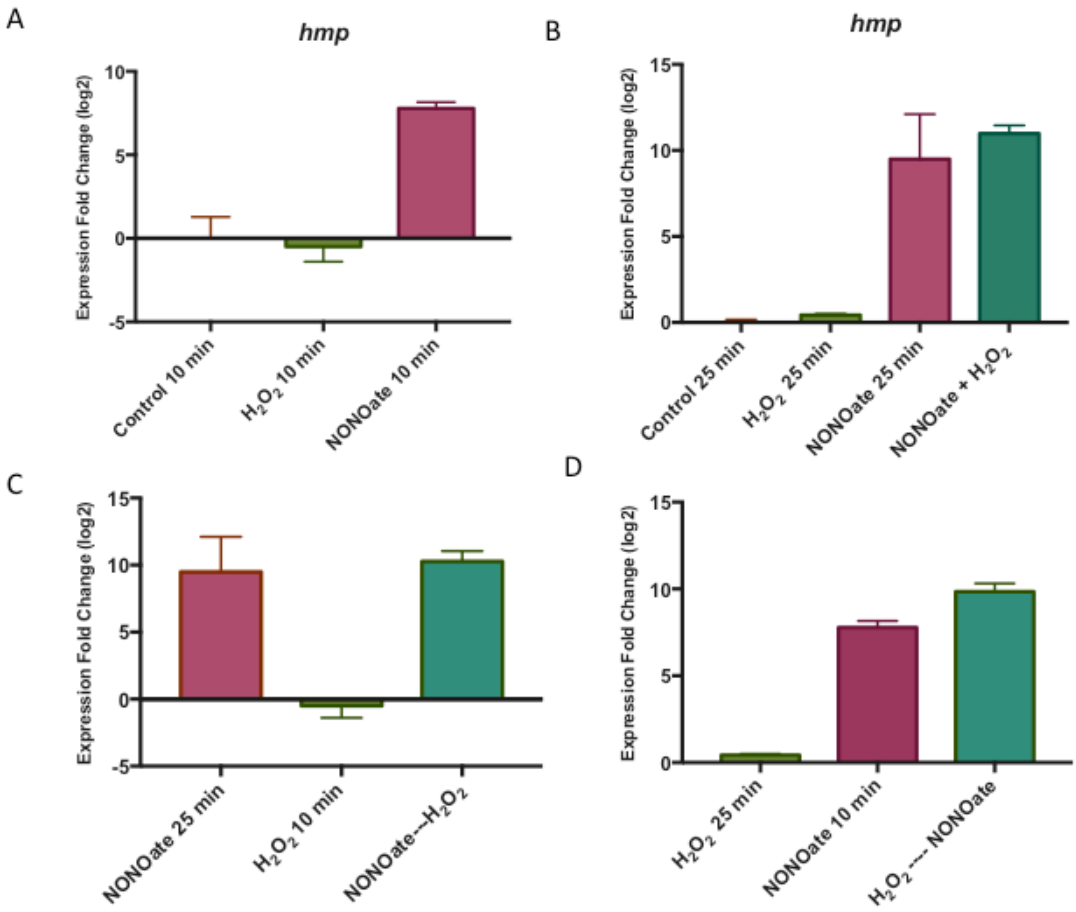


Figure 4.6 Real Time PCR of *hmp* upon sequential exposure to DETA NONOate and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated with the first stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

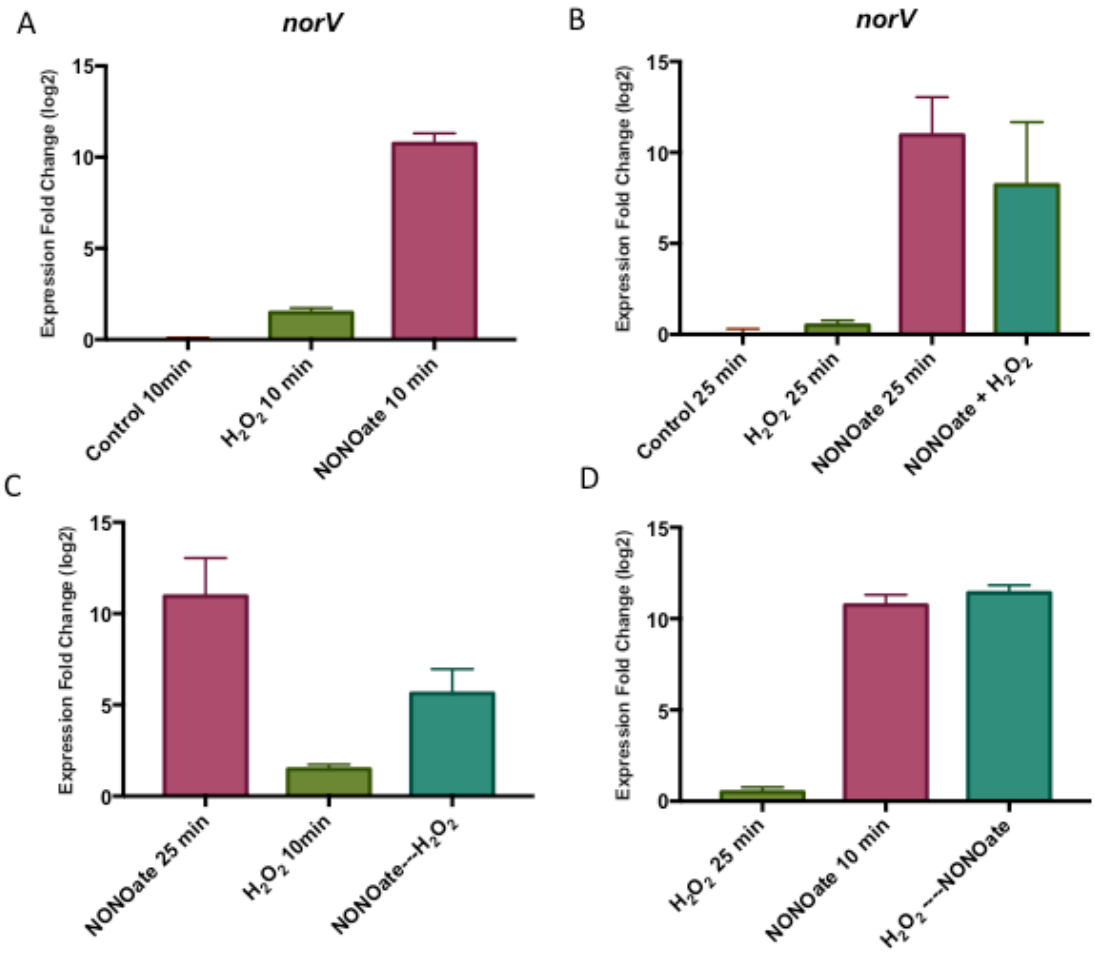


Figure 4.7 Real Time PCR of *norV* upon sequential exposure to DETA NONOate and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated with the first stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

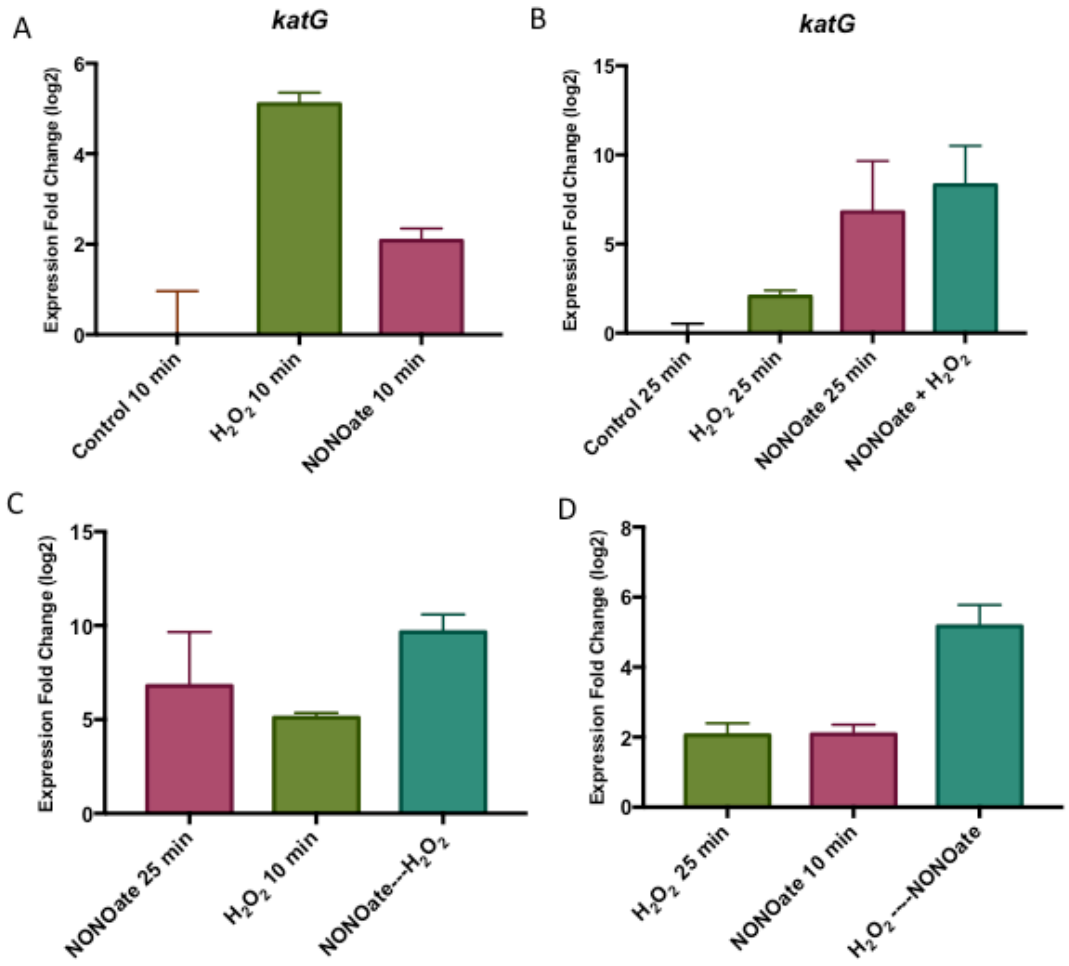


Figure 4.8 Real Time PCR of *katG* upon sequential exposure to DETA NONOate and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated with the first stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

Initial treatment of the cells with DETA NONOate (10 min) then H_2O_2 (15 min) caused an increase in *katG* expression by 800-fold (Fig. 4.8 C). When cells were treated with H_2O_2 (10 min) first then DETA NONOate (15 min), the expression *katG* was up-regulated (40 fold) compared to unstressed control (Fig. 4.8 D).

AhpC has the same extent in responding to nitric oxide and hydrogen peroxide as *katG.* When cells were treated with H_2O_2 for 10 min, the expression of *ahpC* was up-regulated 10-fold and it was up-regulated 4-fold in response to DETA NONOate for 10 min (Fig.4.9 A). When DETA NONOate plus H_2O_2 were applied to the cells for 25 min, the expression of the gene was up-regulated 25 fold (Fig.4.9 B). Exposing the cells to H_2O_2 for 25 min caused no change in the gene expression while exposure the cells to DETA NONOate for 25 min caused up-regulation in the gene expression (19-fold)(Fig.4.9 B). Cells were treated with DETA NONOate first for 10 min then H_2O_2 for 15 min caused up-regulation (48-fold) in the gene expression (Fig. 4.9 C). The expression of the *ahpC* was up-regulated by 8-fold compared to the unstressed control when the cells were treated with H_2O_2 followed by DETA NONOate (Fig. 4.9 D).

As shown in Fig. 4.4 E, *sodA* expression was elevated by reagents other than those that are predominantly generators of superoxide radicals. Thus, when bacteria were treated with H_2O_2 or DETA NONOate alone for 10 min, the expression of *sodA* was increased by 4-fold and 2-fold respectively (Fig. 4.10A). However, on 25 min exposure to H_2O_2, *sodA* expression was marginally down-regulated compared with the unstressed control (Fig. 4.10 B). On the other hand, the expression of the gene was up-regulated by 11-fold with DETA NONOate with 25 min exposure (Fig. 4.10 B).

Exposing the cells to the two stresses simultaneously caused 30-fold up- regulation of *sodA* (Fig. 4.10 B), and treating the cells with DETA NONOate first, followed by H_2O_2, caused the up-regulation of *sodA* (23-fold) compared to an unstressed control (Fig. 4.10 C). The expression of *sodA* was up-regulated 12-fold when cells were exposed to H_2O_2 first then DETA NONOate (Fig. 4.10 D). Thus *sodA* responds in a surprising manner to peroxide and an NO generator. As discussed later, these changes may be triggered by reaction of these stressors with the Fe-S clusters of SoxR.

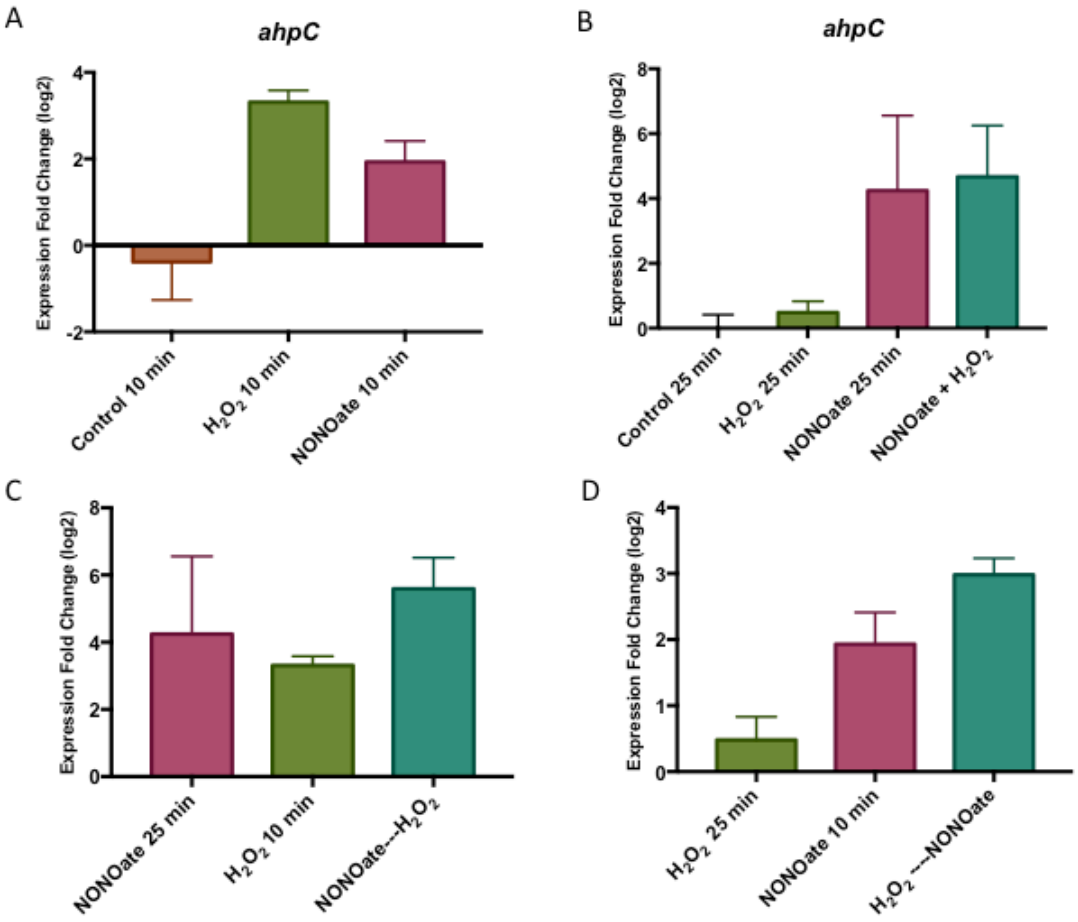


Figure 4.9 Real Time PCR of *ahpC* upon sequential exposure to DETA NONOate and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated with the first stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

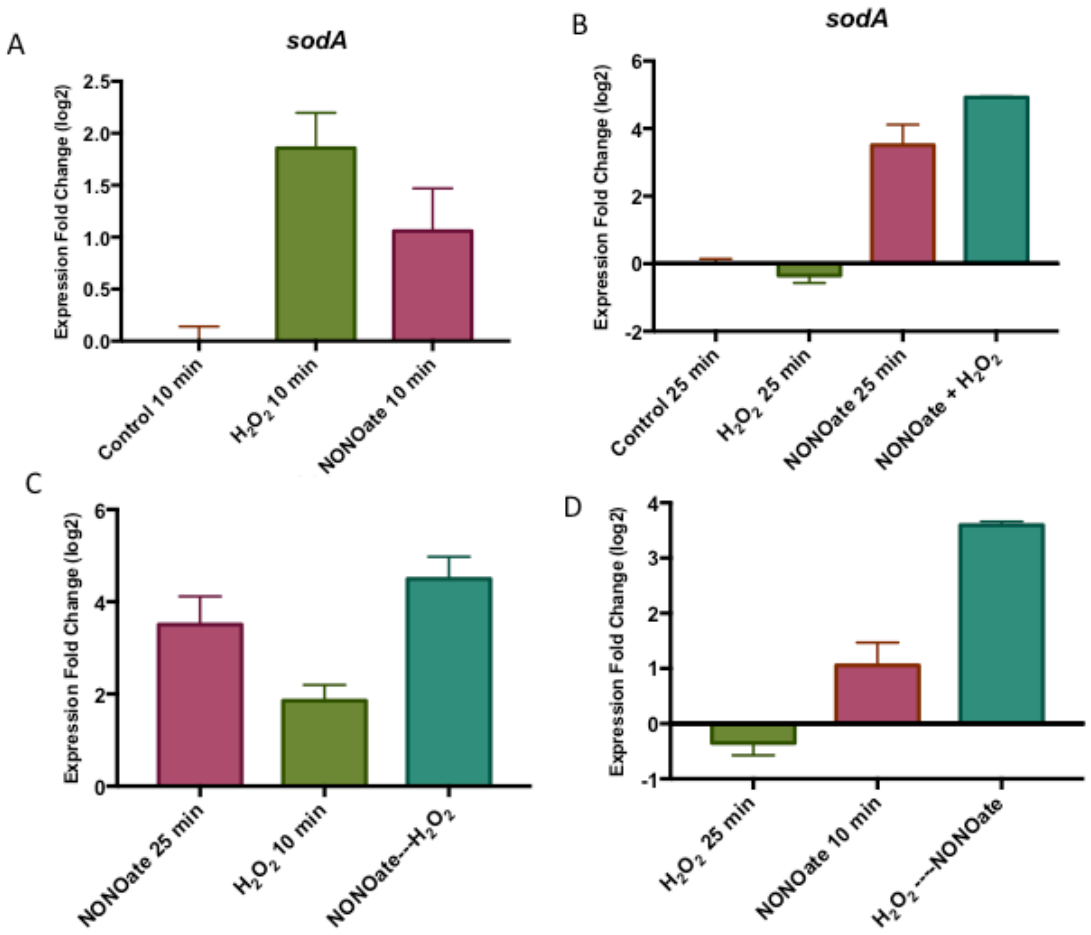


Figure 4.10 Real Time PCR of *sodA* upon sequential exposure to DETA NONOate and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated with the first stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

Figure 4.11 A shows that a 10-min exposure of bacteria to DETA NONOate elicited an 8-fold increase in *nemA* expression, whereas H_2O_2 showed no change in the gene expression. Figure 4.11 B indicates that *nemA* was up-regulated approximately 48-fold responding to DETA NONOate alone (25 min) and 104-fold responding to DETA NONOate plus H_2O_2 (25 min). The expression of *nemA* was upregulated ~2-fold in response to H_2O_2 for 25 min (Fig. 4.11 B). It was also up-regulated 140-fold when cells were treated with DETA NONOate (10 min) then H_2O_2 (15 min) (Fig. 4.11 C). Initial treatment with H_2O_2 (10 min) then DETA NONOate (25 min) caused an up-regulation in the gene expression approximately 70-fold (Fig. 4.11 D).

From these results, we conclude that adding two stresses (H_2O_2 plus DETA NONOate) simultaneously for 25 min caused an up-regulation in the expression of nitrosative and oxidative stresses related gene. The only exception found was in the case of *norV* (Fig. 4.7). However, initial treatment with DETA NONOate followed with H_2O_2 did affect the expression of the *norV* gene. Moreover, the expression of oxidative stresses related genes such as *katG*, *ahpC* and *sodA* were also affected when cells were exposed to H_2O_2 first then followed by DETA NONOate. Exposing the cells with DETA NONOate alone elicits an increase in expression of all 6 genes dependent on treatment time. Surprisingly, the expression of *nemA* was significantly increased when cells were treated with DETA NONOate for 10 or 25 min and the basis of this is unclear.

4.2.4 Real Time PCR of oxidative and nitrosative stress genes upon sequential exposure to DETA NONOate and HOCl

A parallel series of experiments was performed to examine the cumulative and sequential effects of DETA NONOate and HOCl. *E. coli* MG1655 was grown in 10 ml Evans medium and inoculated with 5 % of overnight starter. The growth was recorded every hour at 600 nm using a spectrophotometer. Once the OD reached 0.4, 0.3 mM DETA NONOate and/or 50 μM HOCl were added to the growth for 10 min and 25 min. A sequential exposure experiment was conducted as in section 4.2.3 and demonstrated in Fig. 4.5. Then, a sample was taken for RNA- extraction. RT-PCR was performed on six genes *hmp*, *norV*, *katG, ahpC, sodA* and *nemA*.

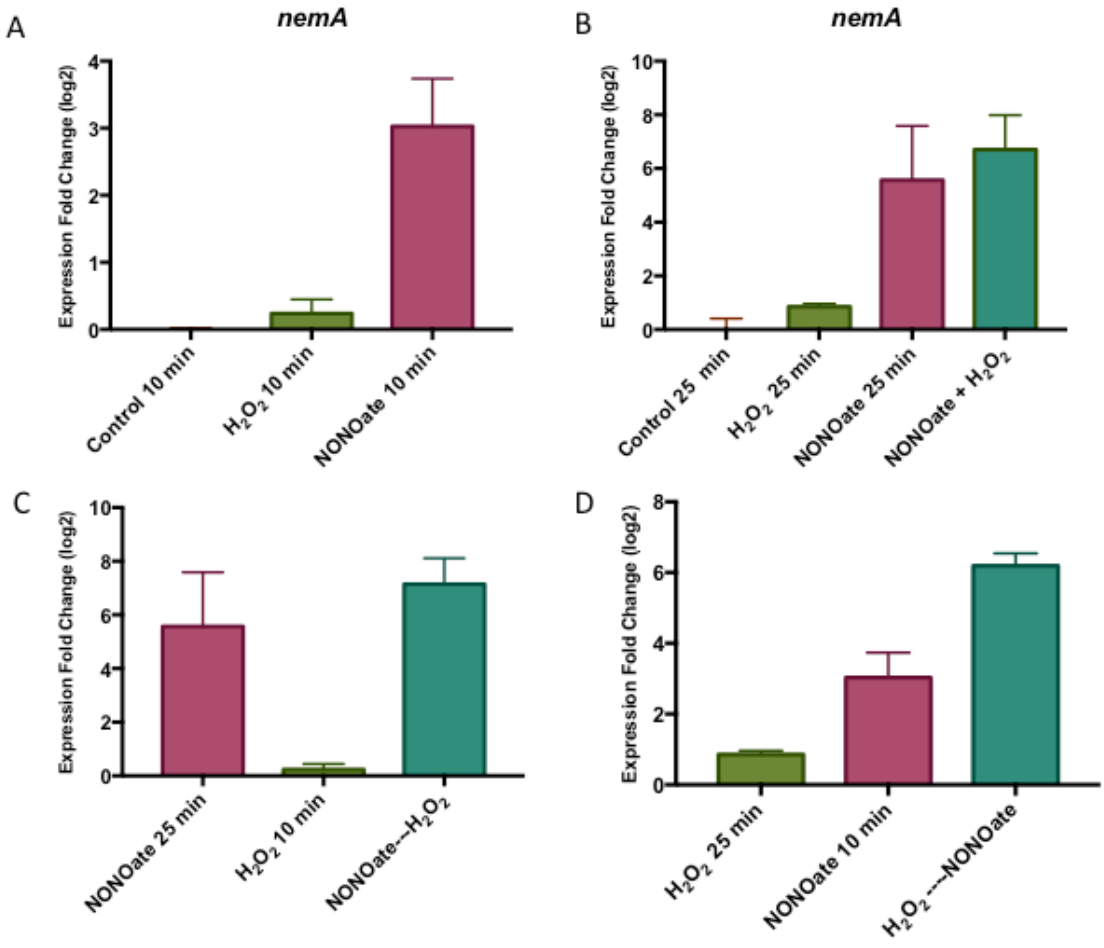


Figure 4.11 Real Time PCR of *nemA* upon sequential exposure to DETA NONOate and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated with the first stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 2 mM H_2O_2 and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

Both genes implicated in nitrosative stresses genes, *hmp* and *norV*, responded somewhat similarly to these treatments (Fig. 4.12 and 4.13). As expected both *hmp* and *norV* were up-regulated by NONOate but HOCl did not significantly change the expression levels, whether it was added with, before or after the NONOate. When HOCl was added with or after the NONOate, there was, however, a modest drop in both *hmp* and *norV* expression. These data suggest that *hmp* and *norV* are responding primarily to the DETA NONOate. Exposing the culture to HOCl first then DETA NONOate caused an up-regulation to *hmp* approximately 630-fold (Fig 4.12 D) and 1782-fold in *norV* expression (Fig 4.13 D).

Neither *katG* nor *ahpC* are expected to respond significantly to HOCl or DETA NONOate *per se*, but either stressor might sense oxidative stress that arises indirectly. For example, inhibition of respiration can promote leakage of reducing equivalents from the electron transfer chain that may react with oxygen to generate peroxides (Imlay, 2013). The *katG* gene was up-regulated >2-fold in response to HOCl (10 min) while *ahpC* was up-regulated ~ 2-fold (Fig. 4.14 A and 4.15 A) suggesting either that *katG* and *ahpC* play roles in the bacterial response to HOCl or involvement of the indirect effects cited. No changes in *katG* and *ahpC* expression was observed when cells were exposed to HOCl for 25 min (Fig. 4.14 B and 4.15 B).

For both genes, HOCl treatment for 10 min caused up-regulation but after 25 min the expression levels reduced. DETA NONOate in contrast gave significant up-regulation at both 10 and 25 min but treatment with DETA NONOate and HOCl gave lower expression levels. Thus, the effects of the stressors are not additive. For both genes, treatment for 25 min with DETA NONOate alone gave higher expression levels than when HOCl was subsequently added (Figs 4.14 C and 4.15 C). In contrast, treatment for 25 min with HOCl alone gave no elevation in expression but when DETA NONOate was subsequently added, the prior presence of HOCl gave higher expression than NONOate alone (Figs 4.14 D and 4.15 D). Thus, the effects of sequential treatments on oxidative gene responses are complex and reveal unexpected interactions.

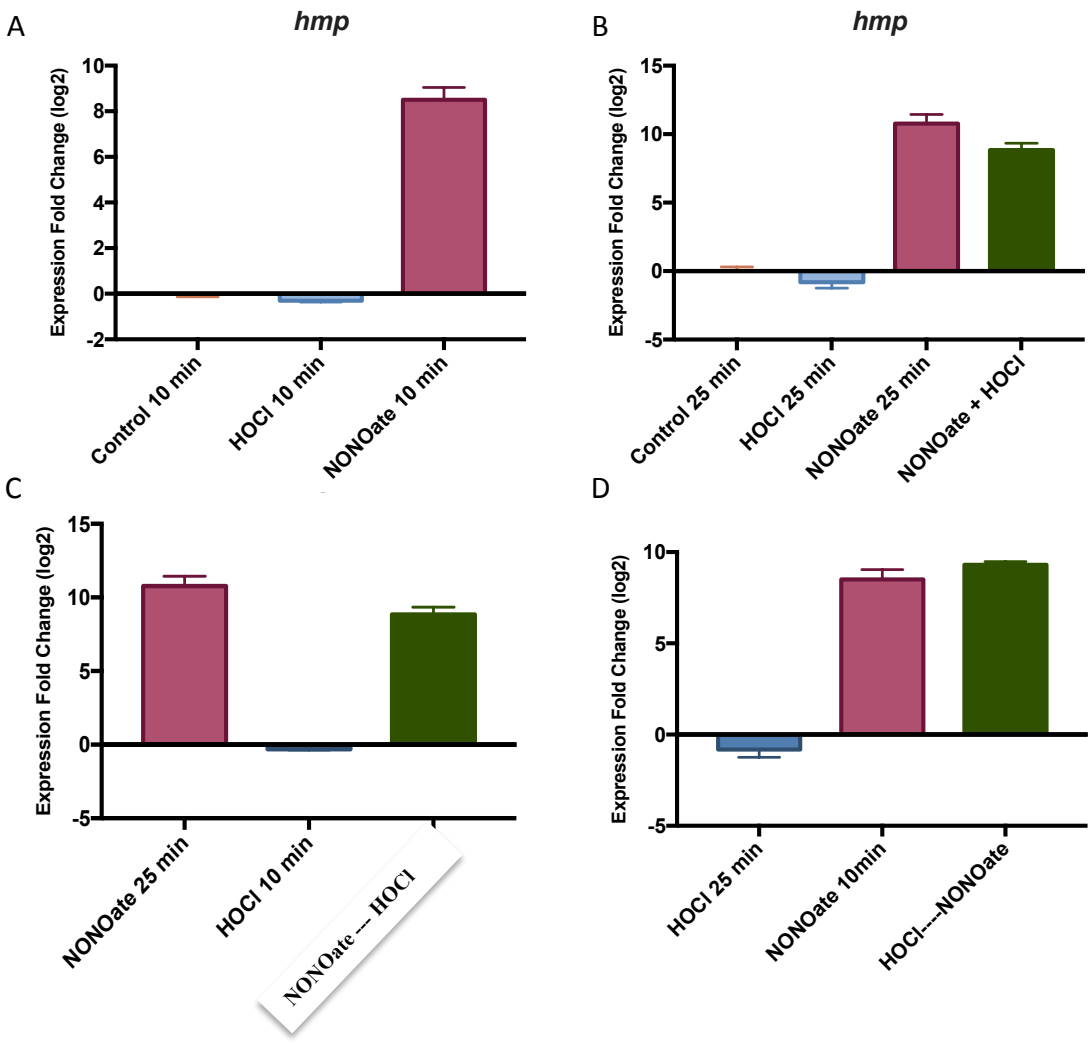


Figure 4.12 Real Time PCR *hmp* upon sequential exposure to DETA NONOate and HOCl

Cultures of *E.coli* MG1655 wild type were treated with first with 50 µM HOCl and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 50 µM HOCl and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total of 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

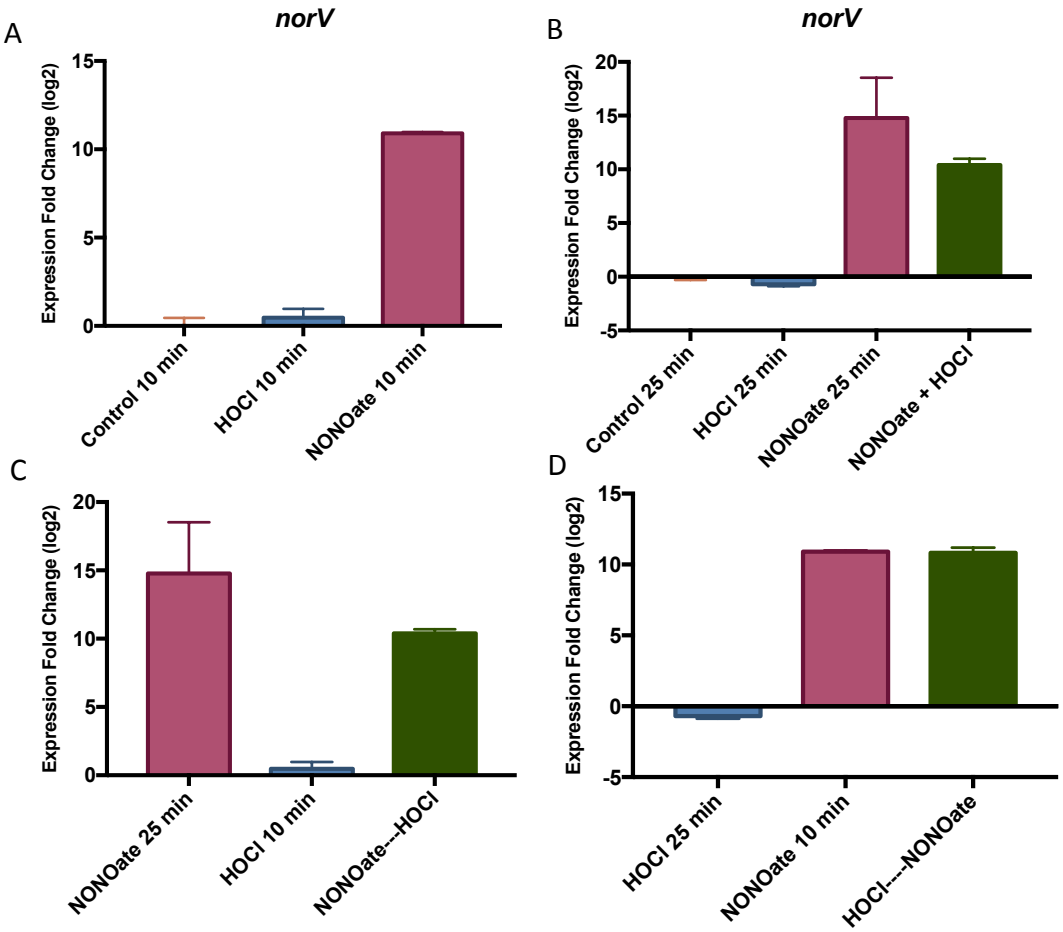


Figure 4.13 Real Time PCR *norV* upon sequential exposure to DETA NONOate and HOCl

Cultures of *E.coli* MG1655 wild type were treated with first with 50 µM HOCl and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 50 µM HOCl and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total of 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

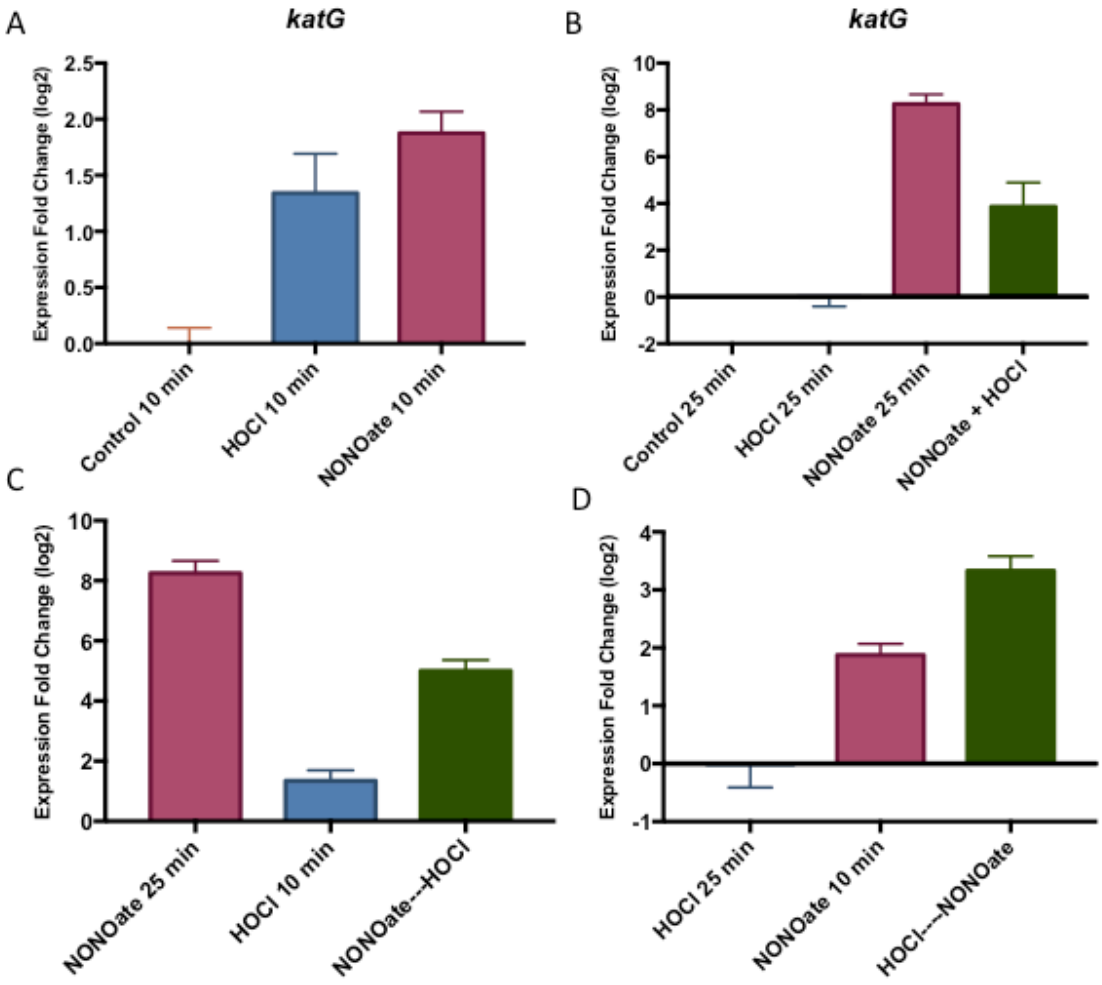


Figure 4.14 Real Time PCR *katG* upon sequential exposure to DETA NONOate and HOCl

Cultures of *E.coli* MG1655 wild type were treated with first with 50 µM HOCl and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 50 µM HOCl and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total of 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

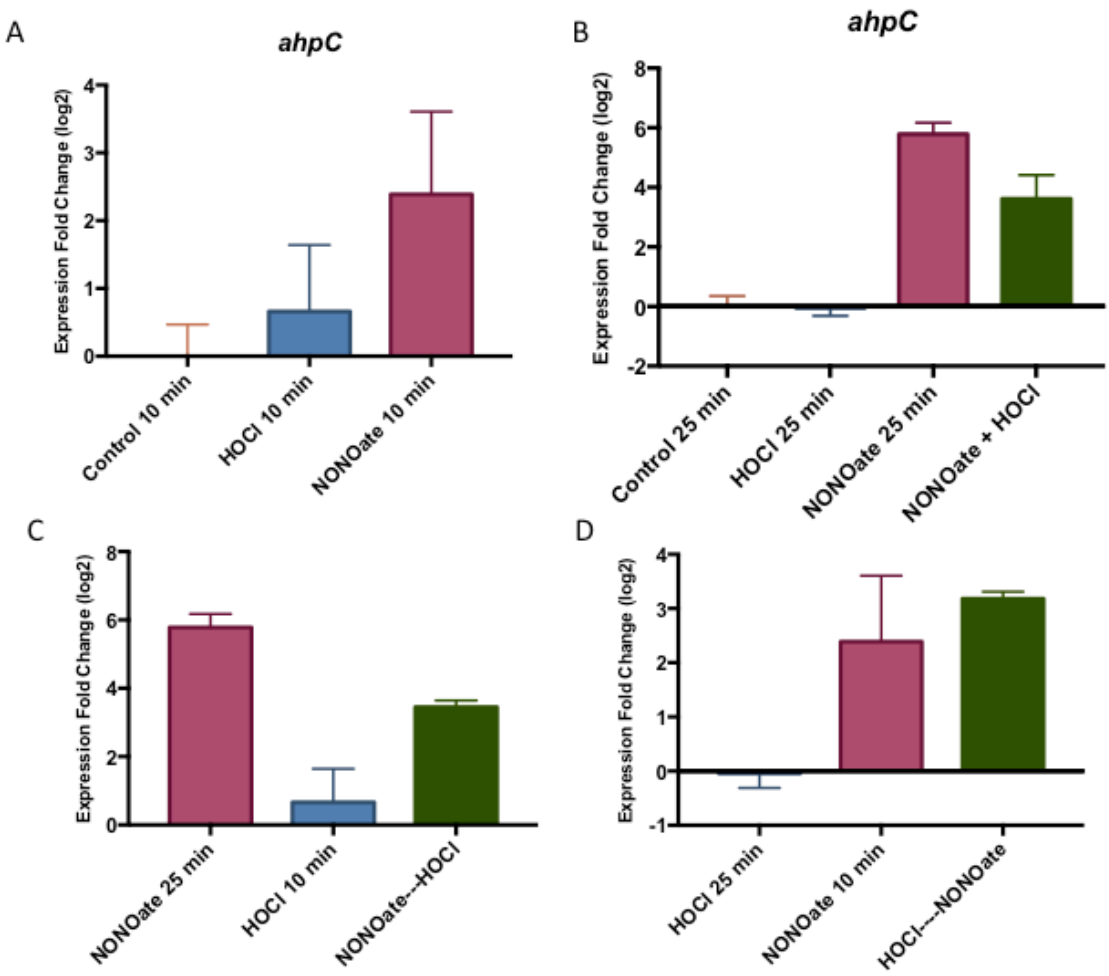


Figure 4.15 Real Time PCR *ahpC* upon sequential exposure to DETA NONOate and HOCl

Cultures of *E.coli* MG1655 wild type were treated with first with 50 µM HOCl and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 50 µM HOCl and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total of 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

The *sodA* gene is not anticipated to respond directly to NONOate or HOCl. However, although HOCl treatment alone for 10 min or 25 min gave low expression levels, DETA NONOate in contrast gave significant up-regulation (25- fold) at 25 min but treatment with NONOate and HOCl gave lower expression levels ~ 8-fold (Fig. 4.16 B). Thus, the effects of the stressors are not additive. Treatment for 25 min with DET NONOate alone gave higher expression levels than when HOCl was subsequently added (Figs 4.16 C). In contrast, treatment for 25 min with HOCl alone gave no significant elevation in expression but when NONOate was subsequently added, the prior presence of HOCl gave high expression levels, approx. 8-fold (Fig. 4.16 D). The effects of sequential treatments on oxidative gene responses are complex and reveal unexpected interactions.

Exposing cells to HOCl for 10 min elicited an approx. 4-fold up-regulation of *nemA* expression (Fig. 4.17 A) while exposing the cells to HOCl for 25 min caused 0.5-fold down-regulation of *nemA* relative to the unstressed control (Fig. 4.17 B). The short-term up-regulation was anticipated due to the role that NemA plays in bacterial survival from HOCl. Down-regulation of gene expression at 25 min was presumably related to the removal of HOCl either as a result of bacterial detoxifying enzyme(s) or its natural instability. Surprisingly, DETA NONOate gave significantly elevated expression of *nemA* after both 10 and 25 min treatment alone. The expression of the gene was up-regulated >50-fold in response to DETA NONOate first then followed with HOCl (Fig 4.17 C) and it was up-regulated ~70-fold when cells were treated with HOCl first then followed with DETA NONOate (Fig 4.17 D). In conjunction with HOCl, its effect was additive. These data and those in which NONOate was added before HOCl or after it (Figs. 4.17 C and D) suggest that NONOate can stimulate *nemA* expression even when HOCl is no longer active.

4.2.5 Real Time PCR of oxidative and nitrosative stress genes upon sequential exposure to NOC-5 NOC-7 and H_2O_2

DETA NONOate has a half-time of NO release of 1260 min at 37 °C (in 0.1 M PBS, pH 7.4) (Aga and Hughes, 2008). Since we observed time–dependent changes in the expression of NO-responsive genes in response to this slow NO releaser (e.g. Figs. 4.6, 4.7, 4.8, 4.12 and 4.13), we selected other NO releasers with different kinetics to

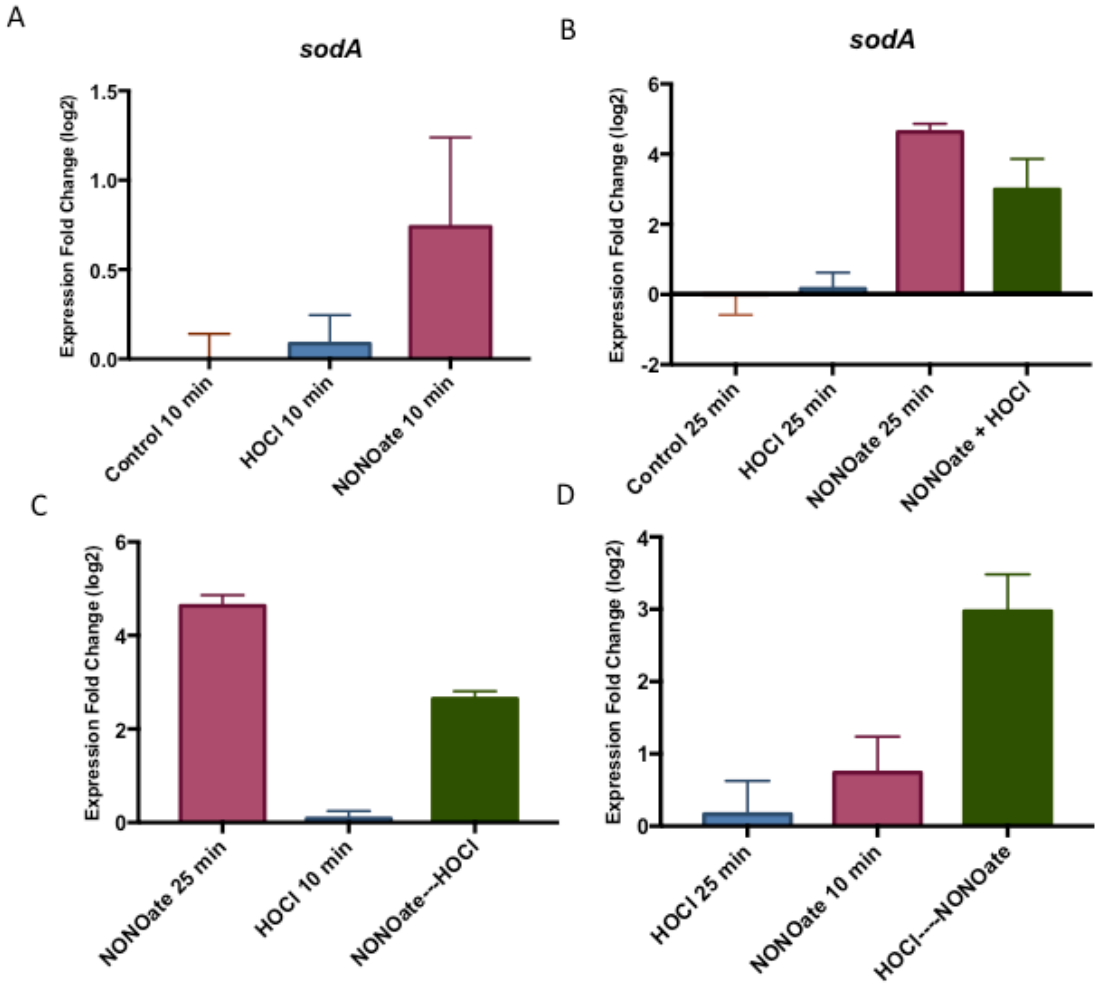


Figure 4.16 Real Time PCR *sodA* upon sequential exposure to DETA NONOate and HOCl

Cultures of *E.coli* MG1655 wild type were treated with first with 50 µM HOCl and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 50 µM HOCl and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total of 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

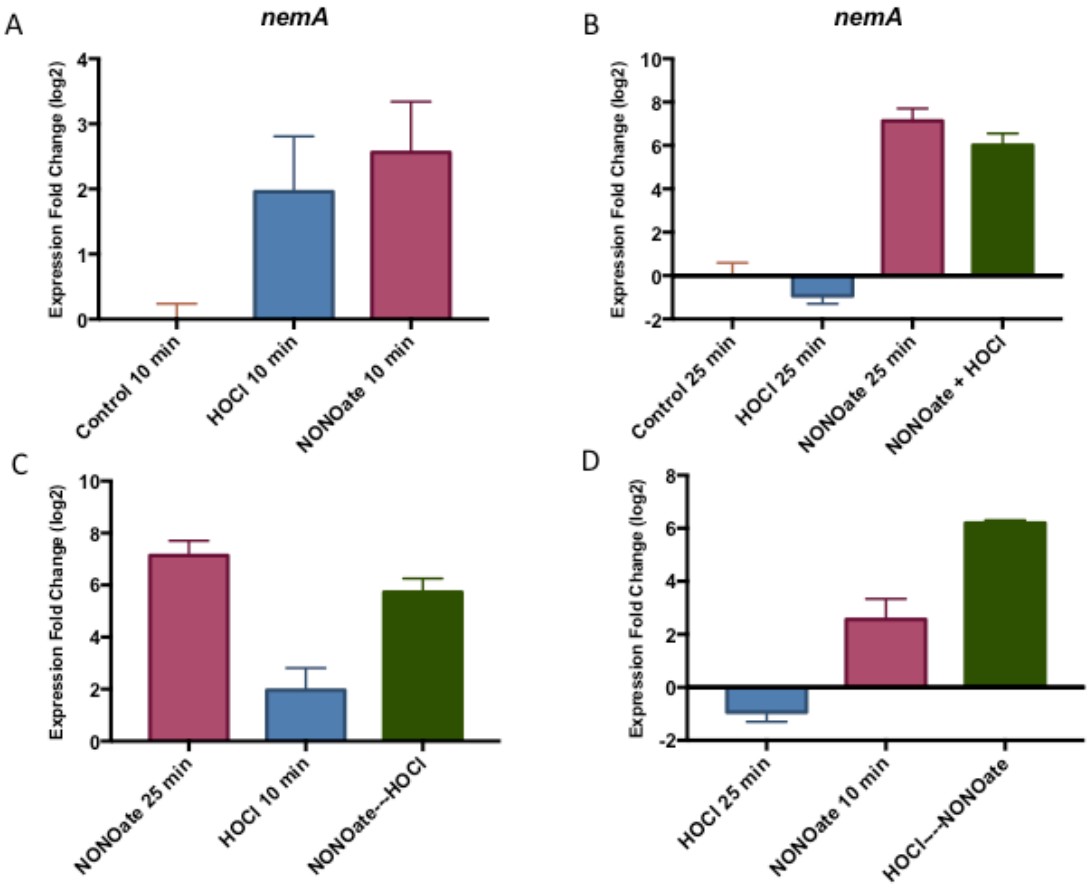


Figure 4.17 Real Time PCR *nemA* upon sequential exposure to DETA NONOate and HOCl

Cultures of *E.coli* MG1655 wild type were treated with first with 50 µM HOCl and/or 0.3 mM DETA NONOate at OD_{600}= 0.4 for 10 min then followed by the second stress 50 µM HOCl and/or 0.3 mM DETA NONOate for 15 min. Some of the cultures were subjected to the stresses for a total of 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

determine whether the observed changes were due to release, then loss, of the NO in solution. We selected NOC-5 and NOC-7 with half-times for NO release of 25 and 5 min, respectively under the above conditions. An equimolar mixture these releasers was used before by Cruz-Ramos et al. (2002).

To investigate how oxidative and nitrosative genes responded to these NO donors in conjunction with other stresses during simultaneous and sequential exposure, *E. coli* MG1655 was grown in 10 ml Evans medium and inoculated with 5 % of overnight starter. The growth was recorded every hour at 600 nm using a spectrophotometer. Once the OD reached 0.4, 0.1 mM NOC-5 plus 0.1 mM NOC-7 and/or 2 mM H_2O_2 were added to the culture for 10 min and 25 min. The sequential exposure experiment was conducted as in section 4.2.3 and demonstrated in Fig.4.5. Then, a sample was taken for RNA- extraction. RT-PCR of *hmp*, *norV*, *katG, ahpC, sodA* and *nemA* was conducted.

When *E. coli* was treated with H_2O_2 alone for 10 or 25 min, there was no change in *hmp* expression compared with the unstressed control (Fig. 4.18 A and B). That confirms the finding in previous results (Fig. 4.6 A). On the other hand, the expression of the gene was up-regulated 388-fold with NOC-5 plus NOC-7 after 10 min exposure (Fig. 4.18 A) and it was also up-regulated 97-fold when cells were exposed to NOC-5 plus NOC-7 for 25 min (Fig. 4.18 B). The increase in the expression of *hmp* in response to NOC-5 plus NOC-7 was expected and the lower expression after 25 min may be due to the decay of NO. In contrast, *hmp* expression increased from 10 to 25 min in Fig. 4.6. Exposing the cells to the two NO donors and H_2O_2 simultaneously gave lower expression (80-fold) after 10 min and 75-fold after 25 min than the NO donors alone (Fig. 4.18 A and B). Treating the cells with NOC-5 plus NOC-7 first then followed with H_2O_2 induced the up-regulation of *hmp* of 55-fold compared to an unstressed control (Fig.4.18 C), a small decrease compared with the NO donors alone. However, the expression of *hmp* was significantly up-regulated 200-fold when cells were exposed to H_2O_2 first then NOC-5 plus NOC-7 (Fig.4.18 D).

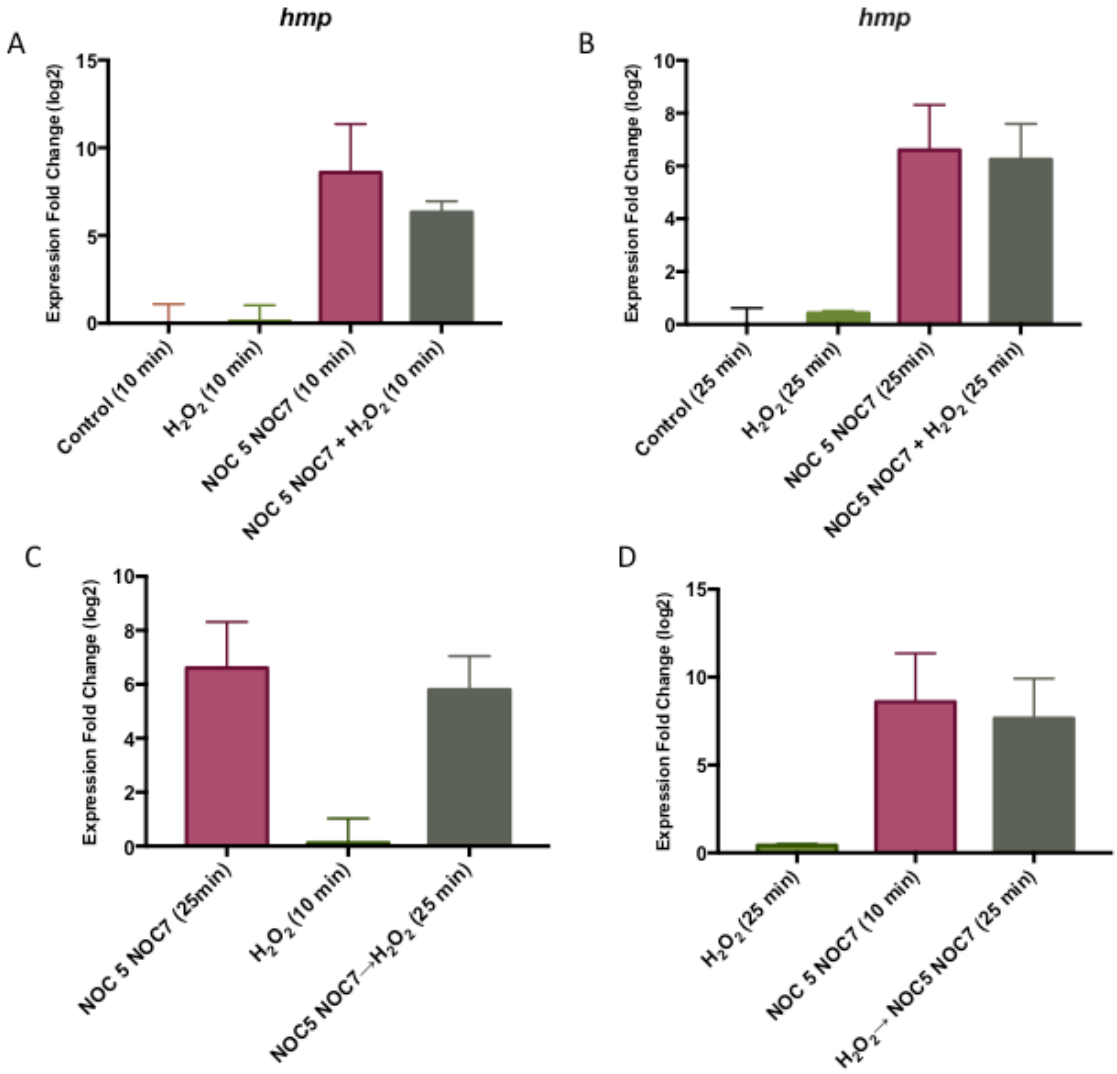


Figure 4.18 Real Time PCR of *hmp* upon sequential exposure to NOC-5, NOC-7 and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated first with 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 at OD_{600}= 0.4 for 10 min followed by the second stress 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

Compared with an unstressed control, the expression of *norV* was significantly increased ~ 720-fold in response to NOC-5 plus NOC-7 after 10 min (Fig. 4.19 A) but it was up-regulated only 82-fold when cells were treated with NOC-5 plus NOC-7 for 25 min (Fig. 4.19 B). This presumably reflects the decay of NO in solution and the failure of the fast NO releaser to adequately replace the NO, as in Fig. 4.18. The expression of *norV* gene was up-regulated 2-fold when cells were exposed to H_2O_2 for 10 and 25 min (Fig. 4.19 A and B), as expected. However, when the NOCs were added together with H_2O_2 (Fig. 4.19A), there was a marked decrease in *norV* up-regulation. A similar result was obtained after 25 min (3.5-fold) (Fig. 4.19 B). One explanation for this is that H_2O_2 reacts with the sensor protein NorR (see Discussion). Further evidence for interaction of H_2O_2 with NO sensing by the *norV* promoter comes from Fig. 4.19 C where H_20_2 added during a 25 min exposure to the NOCs dramatically reduced expression. Similarly, adding H_2O_2 before the NOCs reduced expression relative to exposure to the NOCs alone (Fig. 4.19 D).

Figure 4.20 A and B indicates that *katG* was up-regulated about 35-fold and 3-fold responding to H_2O_2 alone for 10 and 25 min respectively. The gene was also up-regulated about 35-fold when cells were treated to both stresses (NOC-5 plus NOC-7 and H_2O_2) together for 10 min (Fig. 4.20 A) and it was also up-regulated 47-fold when cells were treated with the two stresses for 25 min (Fig. 4.20 B). This result confirms the finding in previous data, which show that *katG* is significantly up-regulated in response to NO plus hydrogen peroxide. There was a 2-fold up-regulation in *katG* expression when cells were treated with NOC-5 plus NOC-7 alone for 10 min and a down-regulation in the gene expression was observed when cells were exposed to NOC-5 plus NOC-7 alone for 25 min (Fig.4.20 B). Initial treatment with NOC-5 plus NOC-7 (10 min) then H_2O_2 (15 min) caused a significant up-regulation in the *katG* gene expression of approximately 70-fold (Fig. 4.20 C); thus the prior presence of the NOCs did not dampen the response to H_2O_2. Similar conclusions can be drawn from Fig. 4.20 D where the order of additions was reversed.

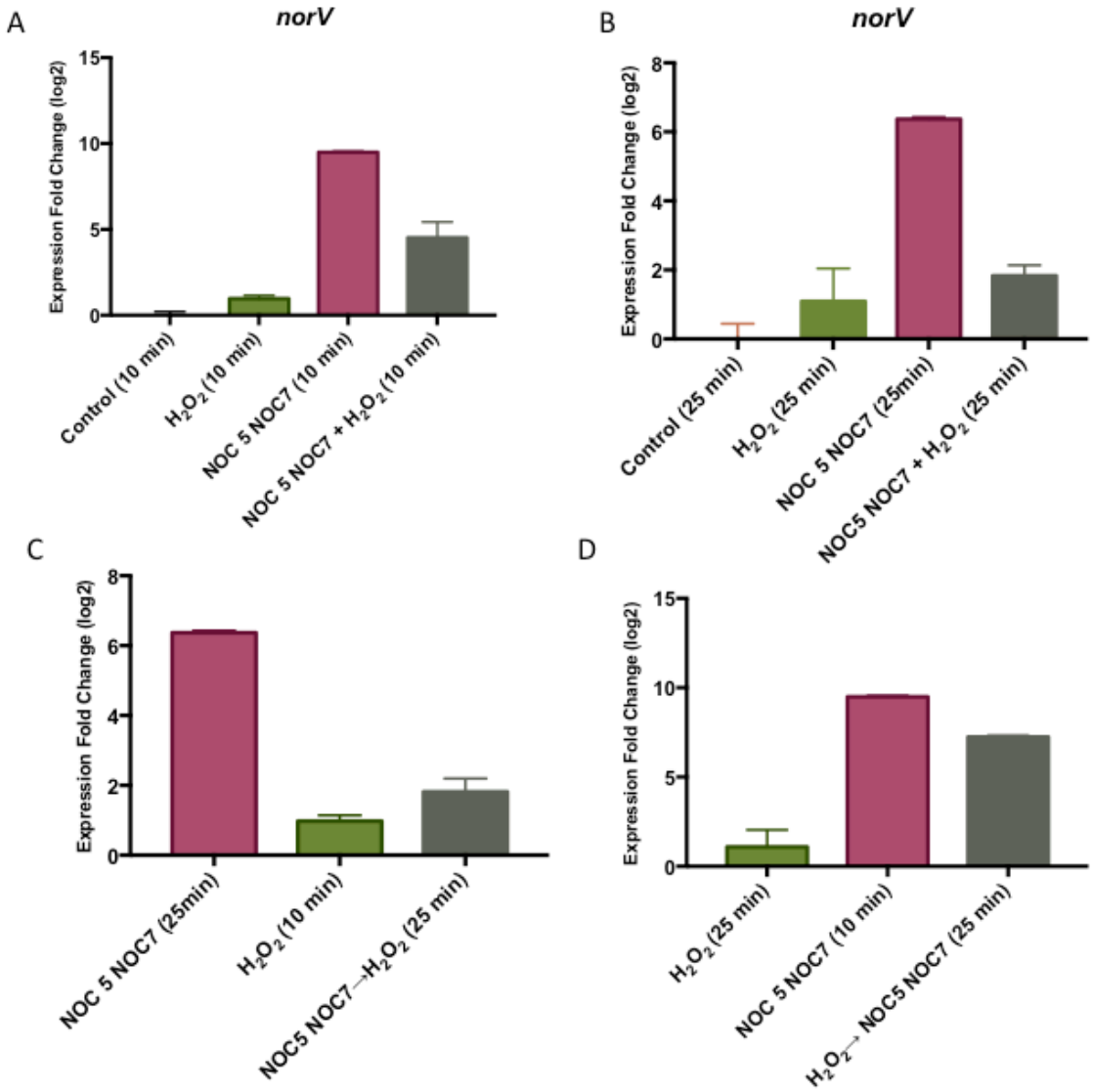


Figure 4.19 Real Time PCR of *norV* upon sequential exposure to NOC-5, NOC-7 and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated first with 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 at OD_{600}= 0.4 for 10 min followed by the second stress 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

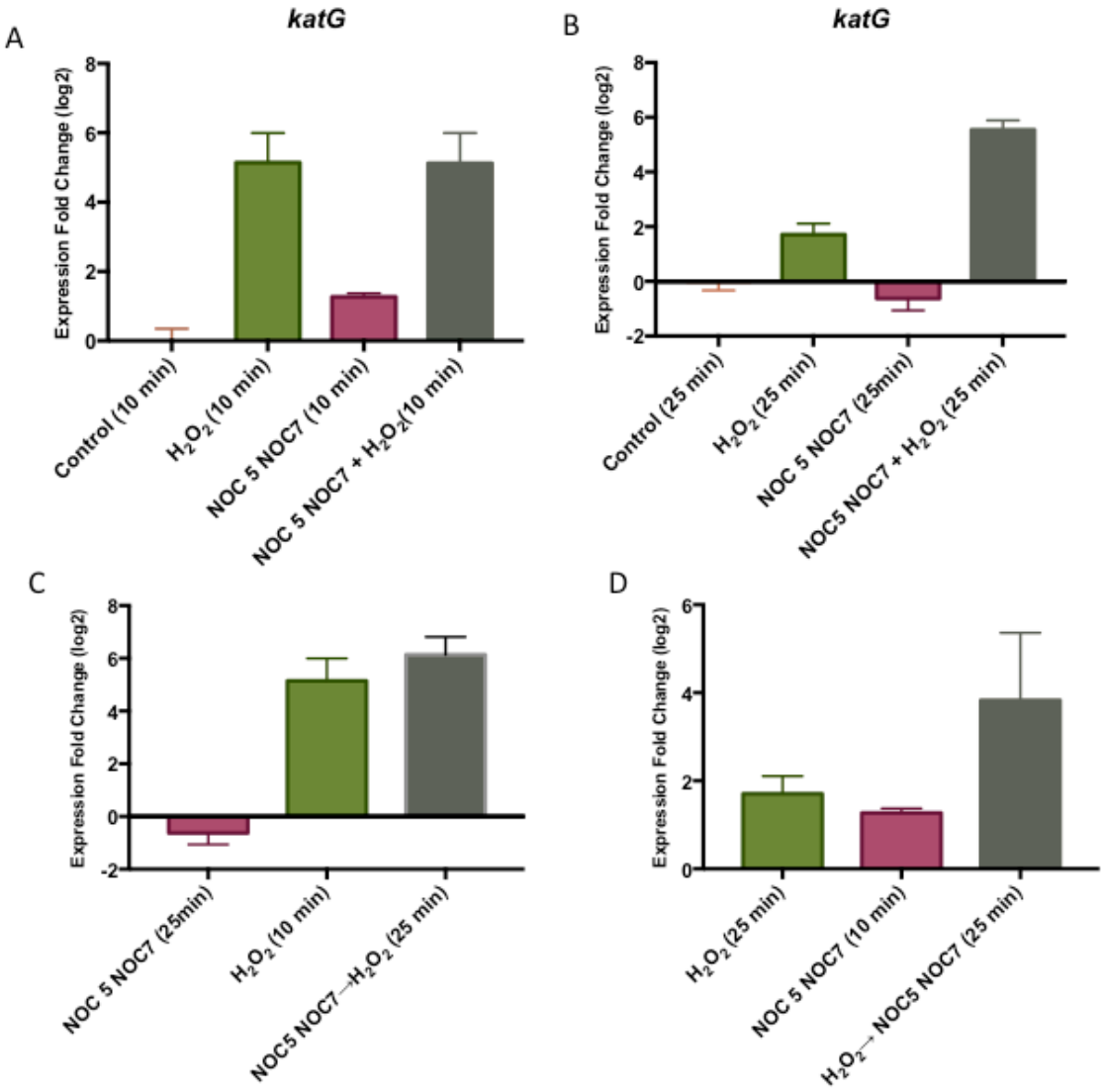


Figure 4.20 Real Time PCR of *katG* upon sequential exposure to NOC-5, NOC-7 and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated first with 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 at OD_{600}= 0.4 for 10 min followed by the second stress 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

In the case of *ahpC*, the NOC compounds alone up-regulated expression (Fig. 4.21 A, B). Exposing the cells to H_2O_2 for 10 min elicited a 9-fold up-regulation of *ahpC* expression (Fig.4.21 A) while exposing the cells to H_2O_2 for 25 min caused no change in the *ahpC* expression relative to the unstressed control (Fig.4.21 B). A decrease in expression upon prolonged exposure to H_2O_2 was also seen in Fig. 4.9 Cells were also treated simultaneously with NOC-5 plus NOC-7 and H_2O_2, which induced the up-regulation of *ahpC* expression by approximately 9-fold in both 10 min and 25 min exposures (Fig.4.21 A and B). As in Fig. 4.9, adding the NO releaser first did not diminish up-regulation by peroxide (Fig. 4.21C). As before, although 25 min prior exposure to H_2O_2 gave little up-regulation, the NO releaser significantly increased expression (Fig. 4.21 D).

Figure 4.22 A indicates that *sodA* was up-regulated about 3-fold responding to H_2O_2 alone for 10 min. No change in gene expression was observed when cells were treated with NOC-5 plus NOC-7 alone or NOC-5 plus NOC-7 combined with H_2O_2 (10 min). The gene was down-regulated 0.7-fold responding to H_2O_2 alone for 25 min or NOC-5 plus NOC-7 for 25 min (Fig.4.22 B). Initial treatment with NOC-5 plus NOC-7 (10 min) then H_2O_2 (15 min) caused an up-regulation in the gene expression of approximately 12-fold (Fig. 4.22 C) while cells were treated with H_2O_2 (10 min) then NOC-5 plus NOC-7 (15 min) elicited approximately 4-fold up-regulation in *sodA* expression (Fig. 4.22 D).

When cells were treated with H_2O_2 alone for 10 min, there was no change in *nemA* expression and it was up-regulated 3-fold responding to NOC-5 plus NOC-7 for 10 min (Fig. 4.23 A). When the two stresses were added simultaneously for 10 min, the expression of *nemA* was up-regulated 4-fold (Fig. 4.23 A), and when the cells were treated with the two stresses for prolonged incubation (25 min), the expression of the gene was up-regulated 6-fold (Fig.4.23 B). This is a further confirmation that *nemA* is responding to NO. Treatment for 25 min with NOC-5 plus NOC-7 alone or H_2O_2 alone caused up-regulation (2-fold) in gene expression levels. The expression of *nemA* was up-regulated 4-fold when cells were exposed to NOC-5 plus NOC-7 first (10 min) then H_2O_2 (15 min: Fig.4.23 C) and it was up-regulated 6-fold when cells were treated with H_2O_2 (10 min) then NOC-5 plus NOC-7 (15 min) (Fig.4.23 D).

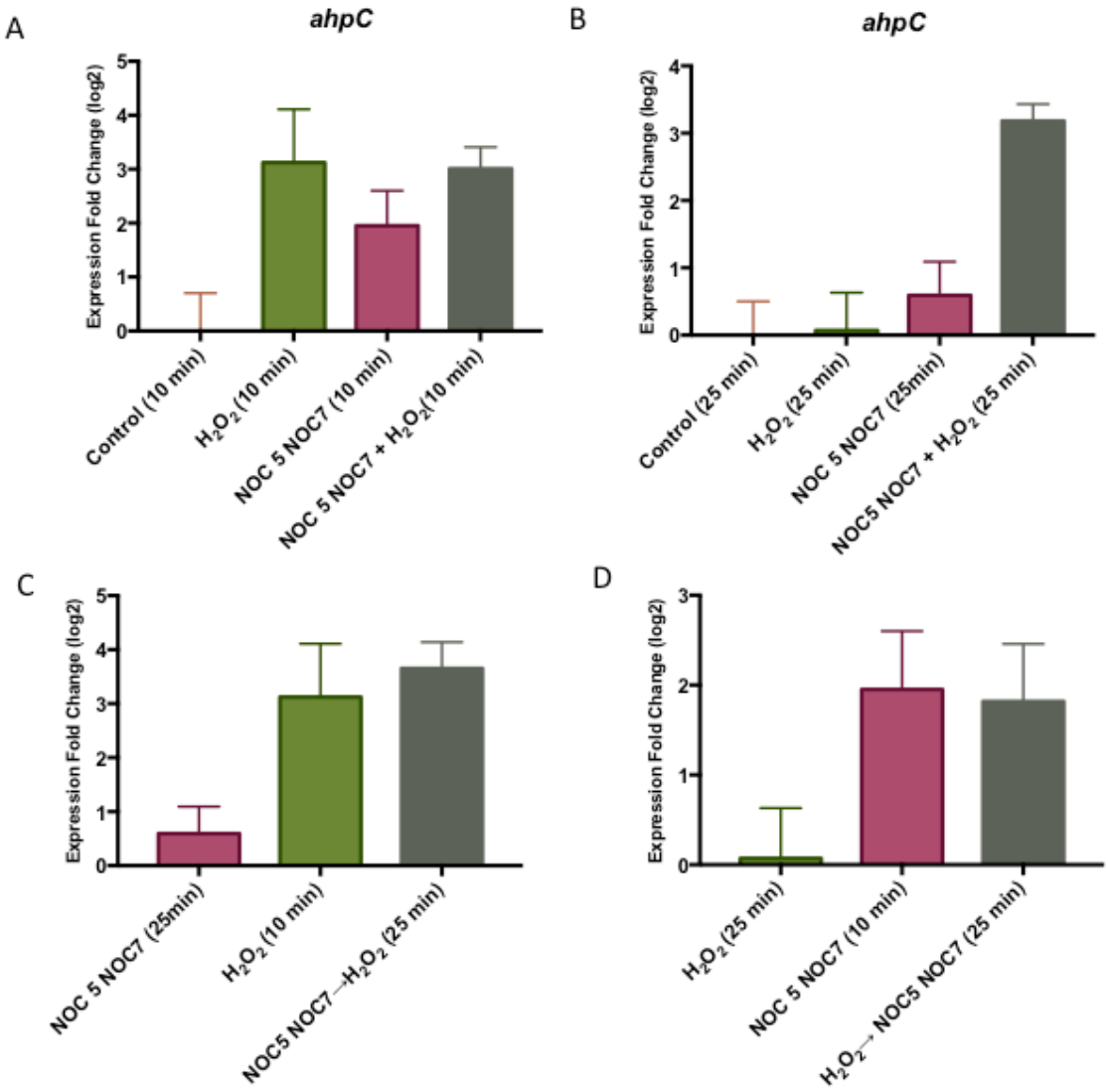


Figure 4.21 Real Time PCR of *ahpC* upon sequential exposure to NOC-5, NOC-7 and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated first with 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 at OD_{600}= 0.4 for 10 min followed by the second stress 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

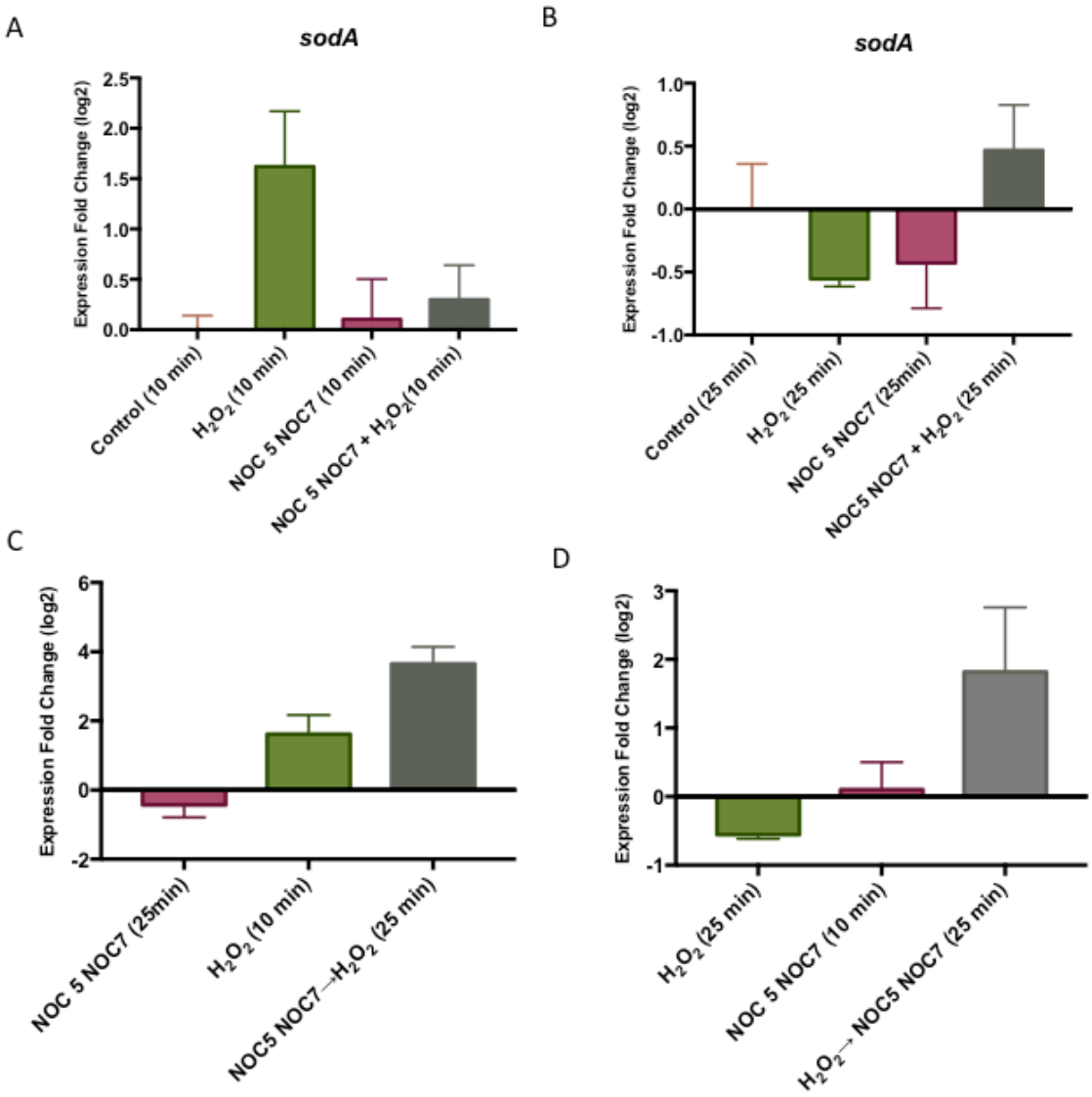


Figure 4.22 Real Time PCR of *sodA* upon sequential exposure to NOC-5, NOC-7 and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated first with 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 at OD_{600}= 0.4 for 10 min followed by the second stress 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

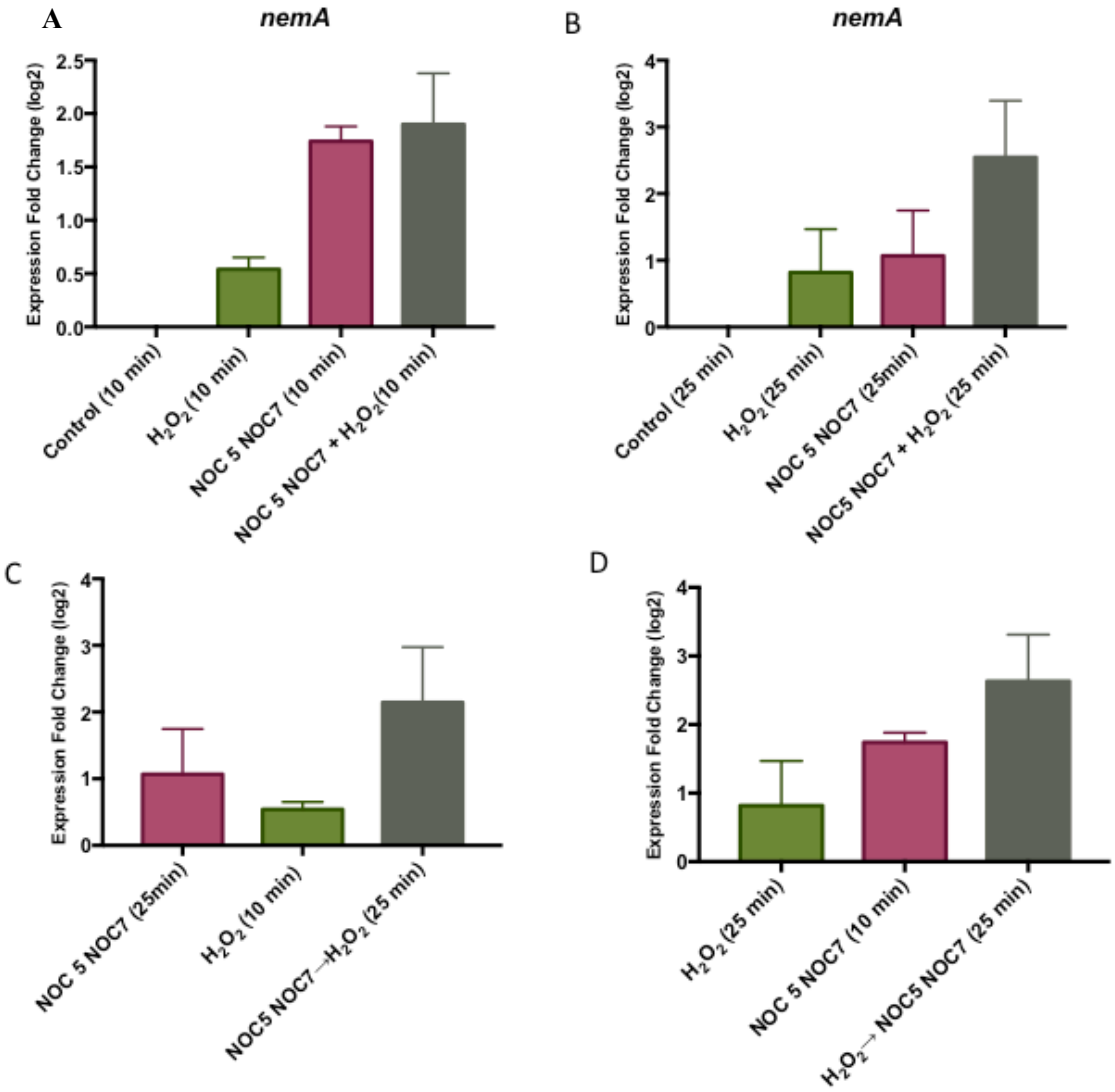


Figure 4.23 Real Time PCR of *nemA* upon sequential exposure to NOC-5, NOC-7 and H_2O_2

Cultures of *E.coli* MG1655 wild type were treated first with 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 at OD_{600}= 0.4 for 10 min followed by the second stress 2 mM H_2O_2 and/or 0.2 mM of NOC-5 plus NOC-7 for 15 min. Some of the cultures were subjected to the stresses for a total 25 min and others for just 10 min. A culture was also untreated as a control. The mean $\log_2$ ratios of individual gene expression relative to the housekeeping gene *gyrA* were then compared with unstressed cells (n=3 ± S.D.).

4.2.6 Real Time PCR of nitrosative and oxidative stress genes upon exposure to inactivated DETA NONOate

In this work we observed a surprising up-regulation of oxidative stress genes seen in the RT-PCR when the cells were exposed to DETA NONOate for 25 min (Figs. 4.8, 4.9, 4.10 and 4.11 B). We therefore investigated whether these transcriptional changes were related to the DETA NONOate compound itself after NO release, or to the NO. For this related experiment, we used inactivated DETA NONOate, which is DETA NONOate left at room temperature for 5 days after bubbling with nitrogen gas for 1 min.

In order to investigate how oxidative and nitrosative stress related genes were responding to inactivated DETA NONOate, *E. coli* MG1655 was grown in 10 ml Evans medium and inoculated with 5 % of overnight starter. The growth was recorded every hour at 600 nm using spectrophotometer. The cells were treated with DETA NONOate or inactivated DETA NONOate. After treating the cells with stresses, cells were incubated for 25 min. Samples for RNA isolation were removed into RNA protect (Qiagen). Adding 0.3 mM DETA NONOate significantly up-regulated all genes in response to oxidative and nitrosative stress (Fig. 4.24). These results correspond to the previous experimental results. However, no change was observed in *katG*, *ahpC* and *nemA* expression when cells were treated with inactivated DETA NONOate while *hmp* and *norV* were up-regulated at a small level (Fig. 4.24). We can therefore conclude that it is NO released from DETA NONOate that is responsible for the elevated gene expression seen.

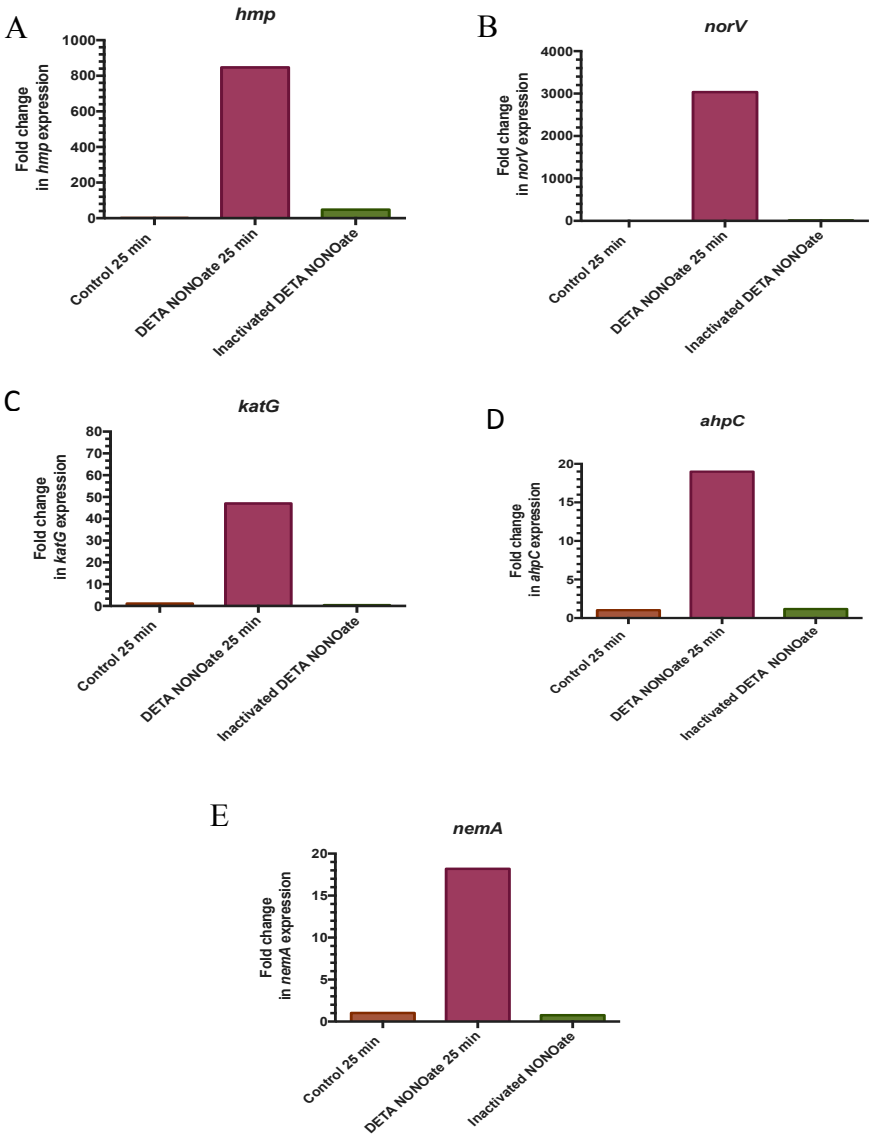


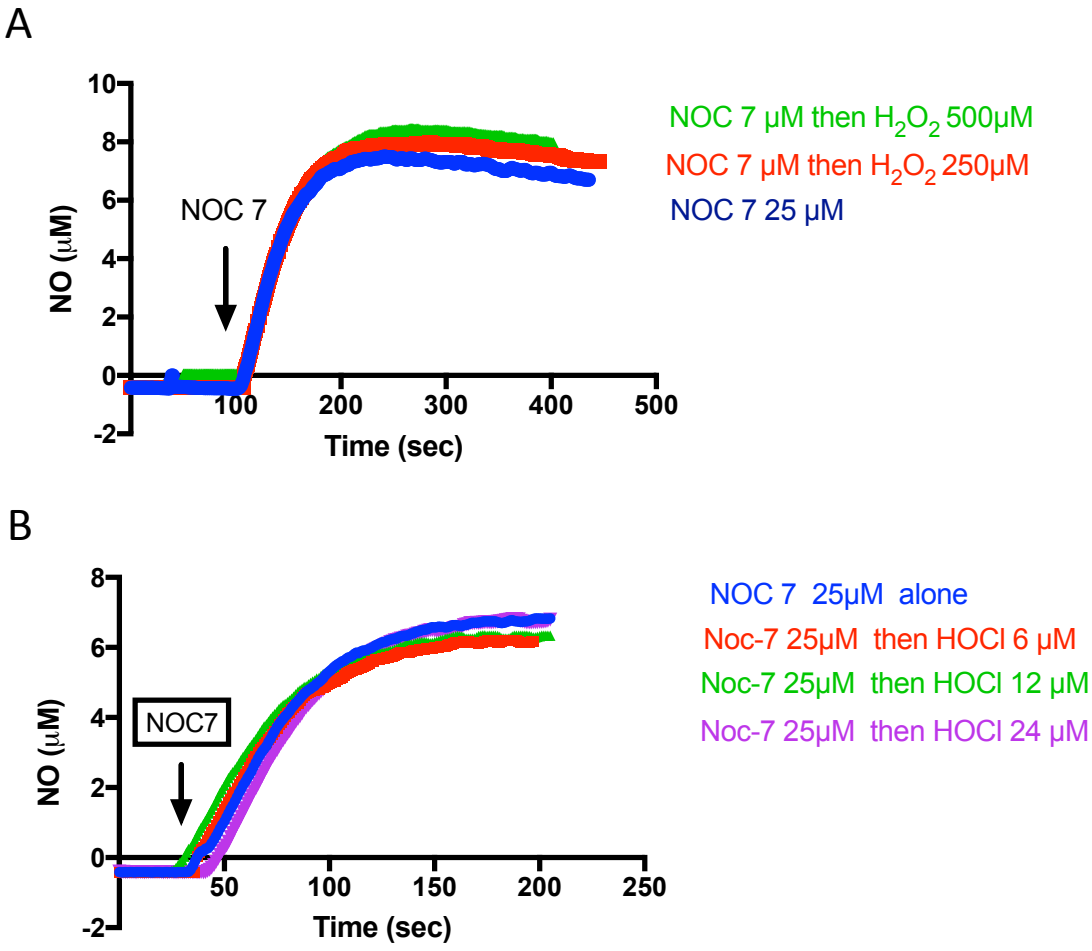
Figure 4.24 Real Time PCR of oxidative and nitrosative stress genes upon exposure to inactivated DETA NONOate and DETA NONOate

Cultures of *E.coli* MG1655 wild type were treated with stress at OD_{600} of 0.4 for 25 min. Some of the cultures were subjected to 0.3 mM inactivated DETA NONOate and others to 0.3 mM DETA NONOate for a total 25 min. A culture was also untreated as control. Then, cells were removed to RNAprotect and processed for RT-PCR analysis. The fold change in individual gene expression compared with unstressed cells was calculated (A) *hmp* (B) *norV* (C) *katG* (D) *ahpC* (E) *nemA*.

4.2.7 Investigating the effect of H_2O_2 and HOCl on nitric oxide release from NOC-7 by using the NO electrode

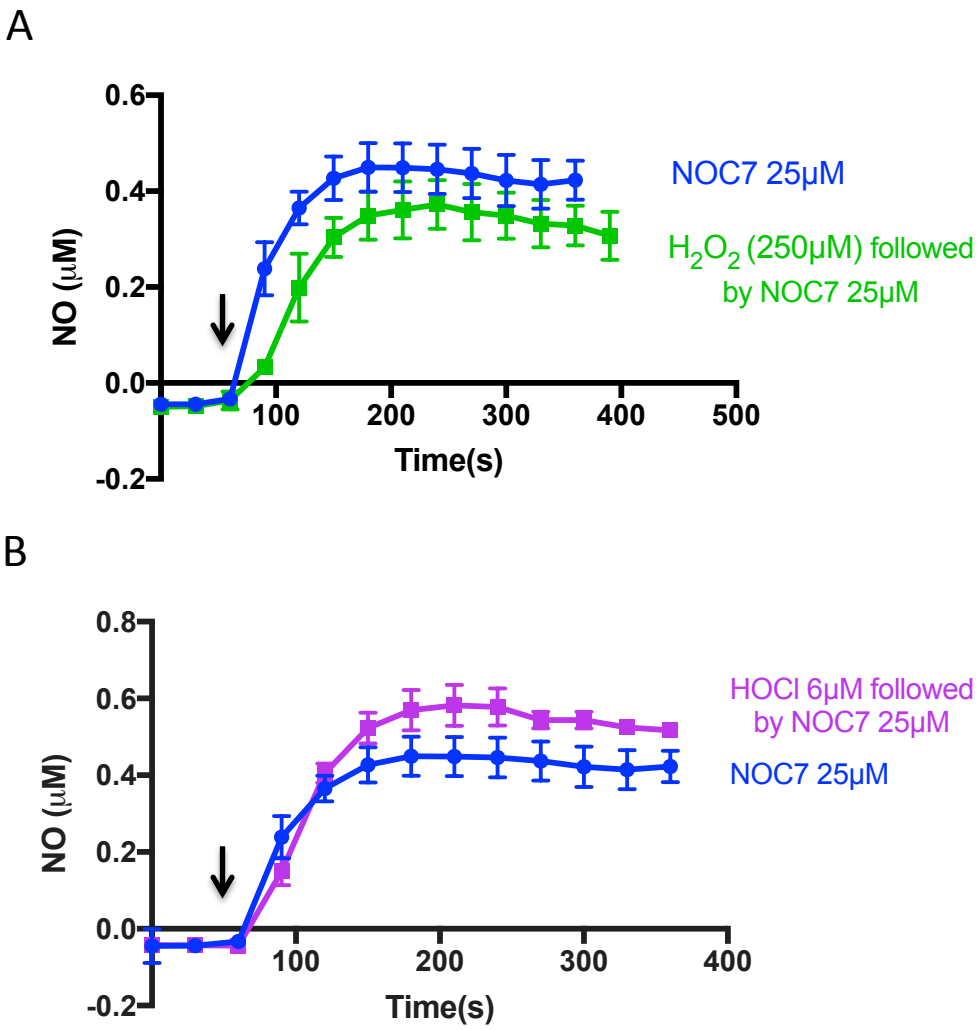
NO is a highly reactive radical. A major route for NO breakdown is reaction with ROS (Kelm, 1999). Thus, chemical changes in NO might occur when NO is combined with H_2O_2 or HOCl and this might influence some of the results obtained. For example, in Fig. 4.7 B, H_2O_2 added after the slow NO release DETA NONOate reduced the expression of *norV* compared to a culture in which only the DETA NONOate was present. Similarly in Fig. 4.14 B, HOCl added after DETA NONOate reduced the expression of *katG* compared to a culture in which only the DETA NONOate was present. We therefore used an NO electrode to determine whether oxidative stress reagents accelerated the loss of NO from solution. An NO electrode was used and inserted into Evans medium with constant stirring. The voltage response was adjusted to settle after a period of 5–10 min. When the baseline voltage of the electrode was stable, NOC-7 (25 μM) were added to the Evans followed by (250 or 500 μM) H_2O_2 or (6, 12 or 24 μM) HOCl and the voltage response was recorded for 6 min (Fig 4.25). The experiment was repeated by adding H_2O_2 (250 μM) or HOCl (6 μM) to Evans medium first then followed by NOC-7 (Fig 4.26) to study the impact of oxidative stress on the NO released from NOC-7.

Figure 4.25 A shows that no changes in either the rates of NO evolution from NOC-7 or the life-time of NO in solution could be detected after additions of H_2O_2 (Fig. 4.25 A + 4.26 A) or HOCl (Fig.4.25 B + 4.26 B). In conclusion, the addition of H_2O_2 or HOCl, even at high concentrations of the two compounds, did not affect NO chemistry. We conclude that the simultaneous and sequential additions of an NO releaser with oxidative stress reagents are not confounded by NO reactions.



4.25 Effects of H_2O_2 and HOCl on nitric oxide release from NOC-7

NO production from adding 25 μM NOC-7 to Evans medium (A) NOC-7 (25 μM) were added first then followed by H_2O_2 (250 and 500 μM).(B) NOC-7 (25 μM) were added first then followed by HOCl (6,12 and 24 μM). Arrows indicates NOC-7 addition to Evans medium. Each addition was preformed after the medium reached the electrode baseline.



4.26 Effects of H_2O_2 and HOCl on nitric oxide release from NOC-7

NO production from adding 25 μM NOC-7 to Evans medium (A) H_2O_2 (250 μM) were added first then followed by (NOC-7 (25 μM). (B) HOCl (6 μM). were added first then followed by NOC-7 (25 μM). Arrows indicates NOC-7 addition to Evans medium. Each addition was preformed after the medium reached the electrode baseline. Data represented are the mean of 3 repeats ± S.E.

4.3 Discussion

The aim of the work in this chapter was to study the response of stress-related genes after adding key stresses individually and/or simultaneously. The conditions for examining the transcriptional changes have been optimized and it is clear from the results obtained that DETA NONOate (nitric oxide donor) also up-regulates oxidative stress related genes and when cells are exposed to other nitric oxide donor (NOC-5 and NOC-7) for 10 min, oxidative stress related genes were also up-regulated. The increase in the expression of oxidative stress response genes (*katG*, *ahpC*, *sodA*) related to these genes also respond to nitric oxide. The explanation may be that NO stimulates OxyR (a regulator that activates *KatG* and *ahpC*) and it also stimulates SoxRS (a regulator that activates *sodA* gene). OxyR can be S-nitrosylated by reactive nitrogen species and activate *katG* (Vazquez-Torres, 2012, Green et al., 2014). Moreover, the SoxRS regulon can be activated by nitrosylating the [Fe-S] cluster (Ding and Demple, 2000). As result, these regulators can be switched on by NO and activate the genes (Spiro, 2007, Vazquez-Torres, 2012).

SoxR and OxyR are regulators that can activate the genes responsive to oxidative stresses. These genes encode proteins involved in degradation of superoxide, hydrogen peroxide and organic peroxide (Imlay, 2008). A striking increase in expression of oxidative and nitrosative stress response genes was observed when cells were exposed to DETA NONOate for 25 min (Fig 4.6, 4.7, 4.8 and 4.9 B). However, the expression of these genes was up-regulated at a low level when cells were exposed to DETA NONOate for 10 min. These results suggest that the slow and sustain release of nitric oxide from DETA NONOate compounds plays a main role in the increase in gene expression over time. DETA NONOate has a half-life of 20 h at 37°C and pH 7.4. It spontaneously liberates 2 equivalents of NO per mole. Based on these results, an additional experiment was conducted with inactivated DETA NONOate to confirm whether the increase in the gene expression is related to nitric oxide released from DETA NONOate or from the compound itself (Fig 4.24). The data presented in Fig 4.24 shows that there was no change in the gene expression of *katG*, *ahpC* and *nemA*, when cells were exposed to inactivated DETA NONOate. Additionally, the gene expression of *hmp* and *norV* was up-regulated at a low level when cells were exposed to the same inactivated compound. From this result we can

confirm that *katG*, *ahpC* and *nemA* were up-regulated in response to NO that was released from DETA NONOate.

Figures 4.18, 4.19, 4.20, 4.21 and 4.22 indicate the effect of NOC-5 and NOC-7 on the expression of nitrosative and oxidative stress response genes, and from these results we can conclude that exposing the cells to NOC-5 and NOC-7 for 10 min can cause a significant up-regulation in nitrosative stress response genes such as *hmp* and *norV* as well as *katG*, *ahpC* and *nemA*. However, the expression of these genes was up-regulated slightly when cells were exposed to these compounds for 25 min. The explanation of this is that NOC-7 with a half-life of 5 min and NOC-5 with a half-life of 25 min are quick- releasing nitric oxide donors and this might be explained by the fact that Hmp is consuming the NO and consequently there is less NO available for up-regulating the genes over time (25 min).

Flavohaemoglobin, Hmp, and the flavorubredoxin, NorV, are the two major mechanisms that enteric pathogenic bacteria such as *E.coli* and *Salmonella enterica serovar Typhimurium* utilize to detoxify nitric oxide (Poole and Hughes, 2000). RT-PCR of *hmp* and *norV* from *E.coli* following DETA NONOate or NOC-5 plus NOC-7 revealed a significant response to these compounds which is consistent with the response of *E.coli* to NOC-5 plus NOC-7 aerobically in previous studies (Pullan et al., 2007). However, the experiments that have applied in Pullan et al. (2007) were different in their choice of the concentrations of NOC-5 and NOC-7 (10 μM final concentration of each compound) that they used to test the effect of these compounds on the expression of *hmp* and *norV*. Other studies have used microarrays to study the effect of nitric oxide or other nitrosative stress on the *E.coli* transcriptome (Flatley et al., 2005, Mukhopadhyay et al., 2004)These studies were also different in their choice of compounds that cause nitrosative stress, growth conditions and their choice of medium. Although there were differences in the conditions or in the concentrations that they used, all these studies confirm that *norVW* and *hmp* (transcription units) were up-regulated.

When *E.coli* was exposed to H_2O_2 alongside DETA NONOate/NOC-5 plus NOC-7, the expression of *hmp* was up-regulated. This up-regulation in the expression of *hmp* was similar to when cultures were treated to the nitric oxide donor alone (Fig. 4.6 B and 4.18 B). Baptista et al. (2012) found that when *E.coli* was treated anaerobically

with NO and H_2O_2, the expression of Hmp was increased to the same level as when the cells were exposed to NO only. In this work, *E.coli* was exposed to sequential exposure of the three stresses (NO, H_2O_2 and HOCl), and the expression of *hmp* could be controlled by the presence of nitric oxide only. Thus, there was no effect of H_2O_2 or HOCl on the expression of *hmp* gene expression.

As mentioned previously in this chapter, the expression of *norV* was up-regulated in response to DETA NONOate or NOC-5 plus NOC-7. However, the expression of the gene was up-regulated at low levels when hydrogen peroxide was added alongside nitric oxide or when the cells were treated to nitric oxide first followed by H_2O_2. Based upon the findings in figures 4.7 and 4.19 A and C, it is clear that H_2O_2 blocks the expression of *norV* in response to NO. Previous work demonstrates that the oxidation of the iron center of NorR by H_2O_2 can prevent NO ligation and NorR should be in the reduced state to be able to bind to nitric oxide and then induce *norV* expression (Baptista et al*.,* 2012).

Figures (4.14 A) and (4.15 A) show that *katG* and *ahpC* were up-regulated when cells were exposed to HOCl for 10 min. This suggests that HOCl elicits an up-regulation in the genes that respond to oxidative stress and are regulated by OxyR. Furthermore, *katG* and *ahpC* play an important role in bacterial resistance to reactive chlorine species, wild-type *E.coli, Staphylococcus aureus and Helicobacter pylori* are more resistant to HOCl than the *katG* mutant of these strains reviewed by Gray et al. (2013a). Moreover, HOCl inactivate SOD which lead to more superoxide and the up-regulation of genes response to oxidative stress (Gray et al., 2013a).

The expression of *katG* and *ahpC* was markedly increased in response to nitric oxide and hydrogen peroxide due to the increase in oxidative stress, caused by hydrogen peroxide and peroxynitrite. Hmp protein reduce O_2 to produce superoxide anion which reacts with nitric oxide to form peroxynitrite (Poole and Hughes, 2000). McLean et al. (2010b) demonstrated that *katG* was significantly up-regulated in response to peroxinytrite and it also up-regulated to a lesser extent in response to hydrogen peroxide. In the presence of oxidative stress, OxyR Cys^{199} can be oxidized to form sulfenic acid (S-OH) which is the activate form of OxyR (Green et al., 2014). OxyR activate *katG* and *ahpC* and that explain the significant up-regulation in the genes expression responding to H_2O_2.

Figures 4.4 E, 4.10 A and 4.22 A show that H_2O_2 elicited up-regulation in *sodA* expression and that related to the activation of SoxR by oxidation [2Fe-S] cluster via single electron which lead then to activate the genes regulated by SoxR including *sodA* (Ding and Demple, 1997).

The *E.coli* NemR regulator is a transcriptional repressor and redox sensor which responds to HOCl by the oxidation form of HOCl-sensitive cysteine residues. NemR controls the transcription of *nemA* encoding *N*-ethylmaleimide reductase and *gloA* encoding glyoxalase I. These two enzymes are essential to detoxify the reactive electrophiles. Thus, they are responsible in bacterial survival from HOCl (Gray et al., 2013b). Exposing the cells to HOCl stress elevated the production of methylglyoxal. Therefore, bacterial cells expressed enzymes essential in detoxifying methylglyoxal as a HOCl protection mechanism. Additionally, NemA can also protect the cells from HOCl by reduction of reactive chlorines (Hillion and Antelmann, 2015). Exposure *E.coli* to HOCl for 10 min elicits an increase in the expression of *nemA* as shown in figures 4.4 F and 4.17 A. The gene *nemA* was up-regulated in response to HOCl as result of a defense mechanism that bacterial cells utilize to detoxify HOCl.

RT-PCR results of the *nemA* gene revealed that *nemA* is sensitive to treatment with nitric oxide released from either DETA NONOate or NOC-5 plus NOC-7 (Fig 4.4 F, 4.11, 4.17 and 4.23 A and B). In contrast, the study of Gray et al. (2013b) showed that no significant change was occurred to *nemR* regulator gene expression in response to 0.2 mM of diethylamine nitric oxide (DEANO). However, *nemA* encodes the enzyme that degrades *N*-ethylmaleimide and toxic nitrous compounds (Umezawa et al., 2008). This can explain the increase in the *nemA* expression in response to nitric oxide donors.

Chapter 5

Combined effects of nitric oxide and antibiotics on *E. coli.*

Chapter 5. Combined effects of NO and antibiotics on *E. coli.*

5.1 Introduction

In the second half of the 20th century, antibiotics played a main role in clinical medicine and saved a high number of people from bacterial infections which previously caused a major public health threat. However, the end of the 20th century and the beginning of the 21st century witnessed the appearance and spread of pathogenic bacteria that are resistant to antibiotics. Consequently, antibiotic therapy failed as a treatment, especially in intensive care units (ICUs) and then led to thousands of deaths every year (Lee et al., 2013).

Due to the spread of bacteria resistance to antibiotics, combining non-antibiotics with established antibiotics enhance the activity of the two compounds against pathogen bacteria. Ejim et al. (2011) reported synergy between benserazide or loperamide (non-antibiotics) and minocycline antibiotic against the minocycline resistance strain *Pseudomonas aeruginosa.* Another example of combining antibiotics with antimicrobial compound was demonstrated in the study by Tavares et al. (2013). The study found sub-lethal concentration of CORM-2 (CO releaser) enhanced the activity of amoxicillin, clarithromycin, and metronidazole against antibiotic resistant *Helicobacter pylori* strains. Further, macrophages studies revealed that CORM-2 in combination with antibiotics highly decreased the ability of *Helicobacter pylori* to cause infection. An earlier study showed a synergy between CORM-2 and doxycycline, cefotaxime and trimethoprim (Wareham et al., 2016). However, CO gas did not show any effects on antibiotics activity (Wareham et al., 2016). Zemke et al. (2014) found that nitrite in combination with polymyxin or colismathate enhanced *Pseudomonas aeruginosa* killing by inhibiting respiration.

NO is an antimicrobial agent, which has a complex effect on microorganisms. NO and its derivatives can interact with many different targets inside cells including iron-sulfur thiols, lipids and DNA bases and tyrosine residues (Fang, 2004). Bacterial cells utilize several mechanisms such as detoxification enzymes, mutations in molecular targets and efflux pump in order to induce antibiotics tolerance. NO in some circumstances can either enhance the activity of antibiotics or inhibit their activity

(Vazquez-Torres and Baumler, 2016). Jones-Carson et al. (2014) study demonstrated that NO protect *Burkholderia pseudomallei* from β-lactam antibiotics. In contrast, they found that NO produced by macrophages enhanced *Burkholderia pseudomallei* killing by β-lactam antibiotics. Thus, NO enhances *Burkholderia pseudomallei* killing *in vivo* but not *in vitro.* Barraud et al. (2006) study found that 500 nM SNP (NO donor) in combination with tobramycin increased the activity of the antibiotic against *Pseudomonas aeruginosa* biofilms.

Kohanski et al. (2007) suggest that common mechanism that β-lactams, quinolones and aminoglycosides antibiotics utilized to kill bacteria is stimulating the production of hydroxyl radicals through a Fenton reaction. Although these three classes of bactericidal drugs shared the same mechanism in killing bacteria, they have different drug target interactions. The aminoglycoside drug target is the ribosome while DNA gyrase and penicillin-binding proteins are the targets of quinolone and β-lactams, respectively (Kohanski et al., 2007).

Gusarov *et al.* (2009) demonstrated that endogenous NO which is produced from bacterial nitric oxide synthases (bNOS) in *B. subtilis* can protect from reactive oxygen species (ROS) formed by antibiotics to increase the toxicity of the antibiotics. On the other hand, van Sorge et al. (2013) demonstrated that endogenous NO in methicillin-resistant *S. aureus* (MRSA) increased the toxicity of aminoglycoside antibiotics and they found the lack of bNOS increased the ability of the cells to be more resistant to streptomycin and gentamicin. The study suggested that endogenous NO in MRSA affects the activity of antibiotics in different and more complicated manner when compared to *B. subtilis* (van Sorge et al., 2013).

H_2S acts like NO in protecting bacterial cells from the toxic effects of oxidative stress generated by antibiotics (Shatalin et al., 2011). The study of Shatalin et al. (2011) found that adding gentamicin to an *E. coli* H_2S-deficient strain showed a significant reduction in cells growth and make the cells more sensitive to the antibiotics compared to a wild-type strain which was pre-treated with 2,2'dipyridyl to repress the formation of Fenton reaction generated by gentamicin treatment.

Uropathogenic *E. coli* (UPEC) exposed to nitrosative stress have enhanced colonization of the mouse urinary tract (Bower *et al.,* 2009). An increase in iNOS

activity was observed in the bladder and urine of patients who were suffering from urinary tract infections (UTI) (Smith et al., 1994). Svensson *et al.* (2010) found an increase in *hmp* expression in UPEC isolates from patients with urinary tract infections. This finding suggests that UPEC isolates face nitrosative stress inside host cells during the infection and activate the NO detoxifying enzyme flavohaemoglobin.

Based on the results obtained in the previous chapters and the observation of the individual effects of NO on bacterial cells and the combinatorial effects of NO with other stresses such as H_2O_2 and HOCl, it was considered interesting to study the antimicrobial effects of NO combined with antibiotics. Therefore, the aim of this chapter is to investigate the interaction effects of NO with doxycycline, cefotaxime, gentamicin and polymyxin B against multidrug-resistant uropathogenic *E. coli* (EC958).

5.2 Results

5.2.1 The minimum inhibitory concentrations of bactericidal and bacteriostatic antibiotics

In order to establish the minimum inhibitory concentrations (MICs) of doxycycline, cefotaxime, gentamicin, polymyxin B and DETA NONOate on *E. coli* (EC958), the MIC was determined by the broth dilution test. *E. coli* (EC958) was grown in defined minimal medium for 24 h at 37°C. The defined minimal medium was inoculated with 3% (v/v) overnight starter. DETA NONOate was tested at concentrations 0.3, 0.6 and 1 mM. Cefotaxime was tested at concentrations 12.5-400 μg/ml. The concentrations that were used for gentamicin were 0.1-1.6 μg/ml, while doxycycline concentrations were 6-50 μg/ml. Polymyxin B was tested at concentrations 0.2-4 μg/ml. The MIC was defined as the lowest concentration of antibiotic/antimicrobial agent that inhibited the visible growth of bacteria after incubation for 24 h (Fig. 5.1).

The data in Figure 5.1 show the growth of *E.coli* (EC958) with different concentrations of cefotaxime, gentamicin, doxycycline and polymyxin B. Cells treated with 400 μg/ml or 200 μg/ml cefotaxime did not display an increase in OD_{600} post treatment (Fig.5.1 A). Upon addition of 100 μg/ml cefotaxime, a prolonged lag in growth lasting ~ 15 h was observed prior to cell recover. Cefotaxime (50 μg/ml) was also caused a prolonged lag in growth lasing ~ 10 h then cells start to recover whereas 25 and 12.5 μg/ml cefotaxime promoted a small growth inhibitory effect relative to untreated cells (Fig.5.1 A). The results demonstrated that *E. coli* (EC958) is resistant to cefotaxime even at high concentrations such as 50 and 100 μg/ml. The MIC of cefotaxime was 200 μg/ml.

However, *E. coli* EC958 was susceptible to gentamicin; 1.6 μg/ml gentamicin was enough to stop the growth of *E. coli* EC958 while 0.8 μg/ml gentamicin caused a prolonged lag in the growth lasting ~13 h then cells start to recover. On the other hand, 0.4 μg/ml gentamicin caused a slight reduction in bacterial growth. Both 0.2 and 0.1 μg/ml gentamicin did not affect bacterial growth. The MIC of gentamicin for *E. coli* EC958 was found to be 1.6 μg/ml (Fig. 5.1 B).

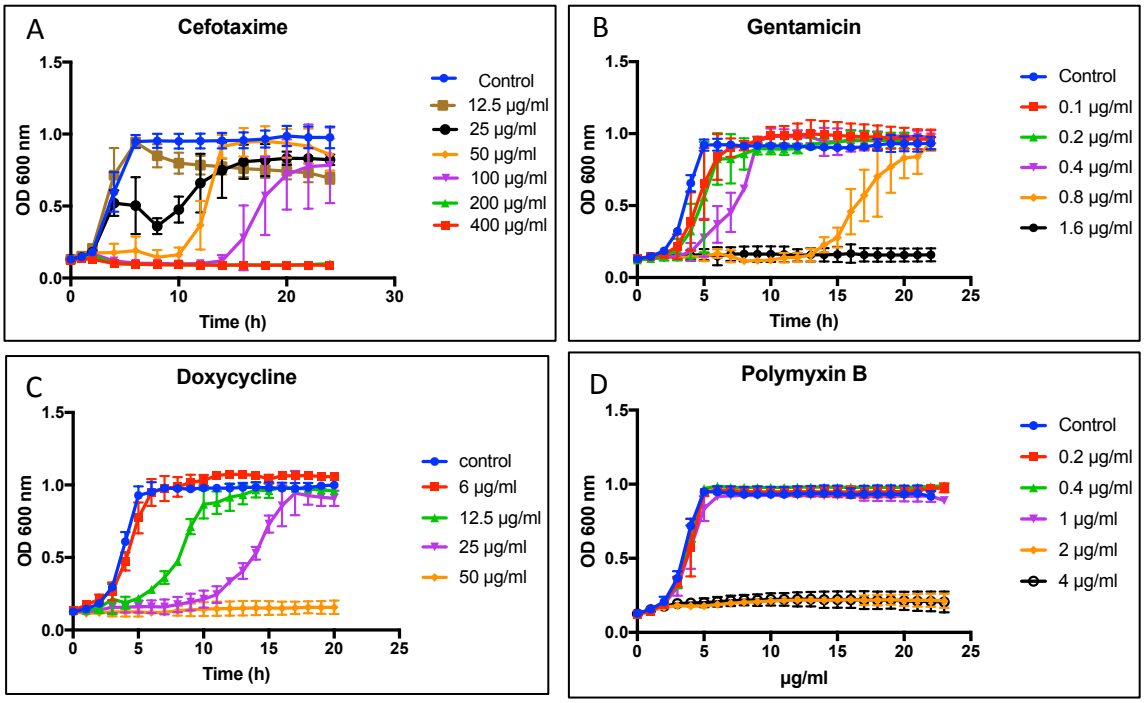


Figure 5.1 The minimum inhibitory concentrations of antibiotics

E. coli EC958 was grown in defined minimal medium for 24 h at 37°C, 200 rpm and OD_{600} was recorded every hour. Antibiotics and DETA NONOate were added at time zero (A) Cefotaxime at concentrations 12.5, 25, 50, 100, 200 and 400 µg/ml. (B) Gentamicin with different concentrations 0.1, 0.2, 0.4, 1 and 1.6 µg/ml. (C) Different concentrations of doxycycline were used; 6, 12.5, 25 and 50 µg/ml. (D) Polymyxin B at concentrations 0.2, 0.4, 1, 2 and 4 µg/ml. Control experiments were carried out in the absence of antimicrobial agents. Data presented are the mean of 3 biological repeats ± S.E

To determine the MIC of doxycycline for *E. coli* EC958, different concentrations of doxycycline was tested. 50 μg/ml doxycycline prevented the growth of *E. coli* EC958 while 25 μg/ml caused a prolonged lag in growth lasing ~ 10 h followed by cell recovery. A prolonged lag was observed lasting a few hours before cell recovery. The concentration of 6 μg/ml doxycycline did not cause any effect on bacterial growth (Fig. 5.1 C).

Cells treated with 2 or 4 μg/ml polymyxin B did not show an increase in OD_{600} after treatment while other concentrations (1,0.4 and 0.2 μg/ml) did not affect the growth (Fig. 5.1 D). The MIC concentration of polymyxin was 2 μg/ml.

The result established that 1 mM DETA NONOate stopped the growth of *E. coli* EC958 after incubation for 24 h (Fig 5.2) while 0.3 mM and 0.6 mM had sub-lethal effects on the growth of *E. coli* EC958. Therefore, the MIC of DETA NONOate for *E. coli* EC958 is 1 mM.

5.2.2 The effects of antibiotics (± DETA NONOate) on bacterial growth

In order to determine the activity of antibiotics alone and in combination with DETA NONOate against antibiotic-resistant *E. coli* EC958, defined minimal medium was inoculated 3% (v/v) with an overnight culture of *E. coli* EC958 then supplemented with different concentrations of cefotaxime, gentamicin, doxycycline and polymyxin B. DETA NONOate (0.2, 0.3 and 0.6 mM) were simultaneously added to different concentrations of antibiotics at the time zero (Figure 5.3). Cultures were grown at 37 ºC, 200 rpm for 24 h then OD_{600} was recorded.

Bacterial growth decreases when cultures were treated with high concentrations of cefotaxime (200 μg/ml) (Fig. 5.3 A). This confirms the resistance of *E. coli* EC958 to cefotaxime as mentioned in section 5.2.1. Adding 0.2 or 0.3 mM DETA NONOate to 25 or 12 μg/ml cefotaxime did not affect bacterial growth while adding same DETA NONOate concentrations to 50 μg/ml caused a slight inhibition in the growth. Treating the cells with 0.2 or 0.3 mM DETA NONOate combined with 100 μg/ml cefotaxime demonstrated a significant growth inhibition (Fig. 5.3 A). This result suggests that DETA NONOate enhances drug efficiency.

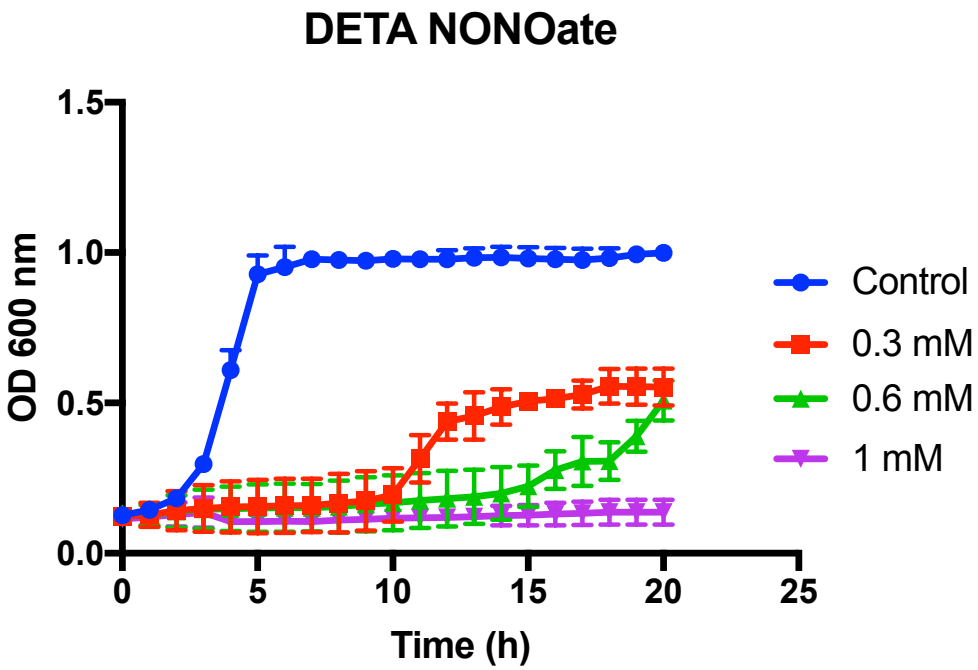


Figure 5.2 The minimum inhibitory concentrations of DETA NONOate

E. coli EC958 was grown in defined minimal medium for 24 h at 37°C, 200 rpm and OD_{600} was recorded every hour. DETA NONOate were added at time zero. DETA NONOate concentrations 0.3, 0.6, 1 mM. Control experiments were carried out in the absence of antimicrobial agents. Data presented are the mean of 3 biological repeats ± S.E

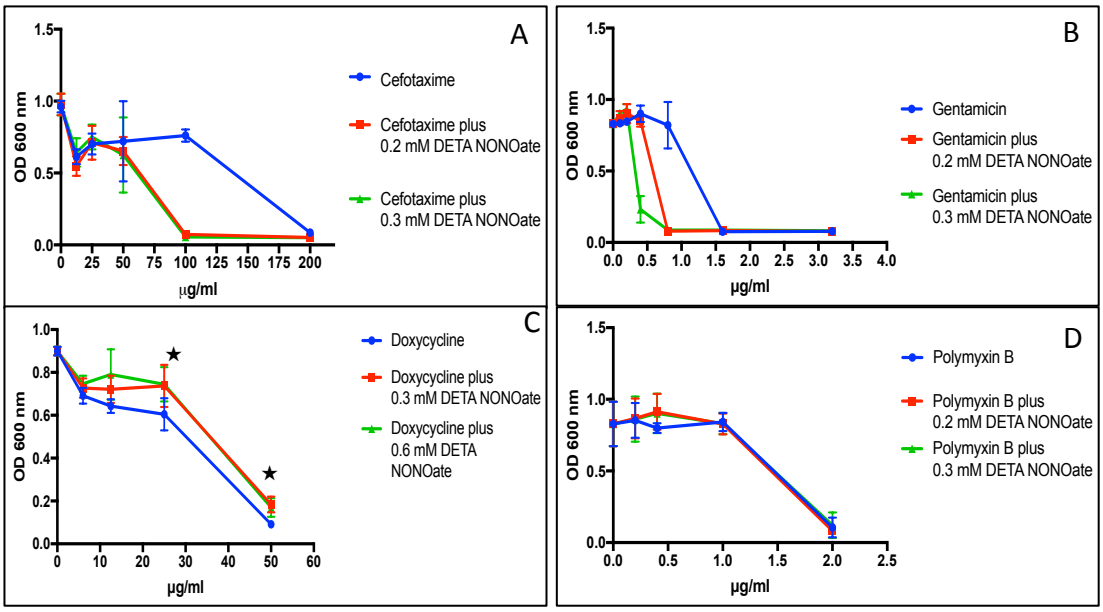


Figure 5.3 The effects of antibiotics (± DETA NONOate) on bacterial growth

E. coli EC958 were grown in defined minimal medium for 24 h at 37°C, 200 rpm and OD_{600} was measured after 24 h. Antibiotics and DETA NONOate were added at time zero (A) Cefotaxime at concentrations 0, 12.5, 25, 50, 100, 200 µg/ml alone or combined with 0.2 mM or 0.3 mM DETA NONOate. (B) Gentamicin at different concentrations 0, 0.1, 0.2, 0.4, 0.8, 1.6 and 3 µg/ml alone or combined with 0.2 mM or 0.3 mM DETA NONOate. (C) Different concentrations of doxycycline 0, 6, 12.5, 25 and 50 µg/ml alone or combined with 0.3 mM and 0.6 mM of DETA NONOate (D) Polymyxin B at concentrations 0, 0.2, 0.4, 1, and 2 µg/ml alone or combined with 0.2 mM or 0.3 mM DETA NONOate. Data presented are the mean of 3 biological repeats ± S. ⋆shows a p value < 0.05 [ANOVA test] for significance of difference to the effect of doxycycline alone.

As mentioned previously, the MIC of gentamicin against *E. coli* EC958 is 1.6 μg/ml. DETA NONOate (0.2 and 0.3 mM) was added to cultures alongside different concentrations of gentamicin. Adding 0.2 mM DETA NONOate combined with 0.1,0.2 or 0.4 μg/ml gentamicin did not affect bacterial growth while treating cells with 0.2 mM DETA NONOate and 0.8 μg/ml gentamicin prevented bacterial growth. Treating the cells with 0.3 mM DETA NONOate in combination with 0.8 μg/ml also prevented the growth of *E. coli* EC958 (Fig. 5.3 B).

Doxycycline (50 μg/ml) was the lowest concentration that prevented growth of the bacterial. However, adding 0.3 mM DETA NONOate combined with different concentrations of doxycycline enhanced the bacterial cells ability to recover from the toxic effects of doxycycline (Fig. 5.3 C). There was a statistically significant increase in bacterial growth when cells were treated with 0.3 mM DETA NONOate plus 25 or 50 μg/ml doxycycline relative to cells treated with doxycycline alone. Combining DETA NONOate (0.3 mM) with 12.5 μg/ml doxycycline also increased the cells growth but it was not statistically significant. Although DETA NONOate (0.6 mM) caused an increase in bacterial growth when it was combined with 12.5, 25 and 50 μg/ml doxycycline, the increase in the growth was not significant (Fig. 5.3 C).

Combination of DETA NONOate with polymyxin B was also tested against *E.coli* EC958. When bacterial cells were treated with DETA NONOate plus different concentrations of polymyxin B, no changes occurred in the activity of the antibiotic (Fig 5.3 D). This result suggests that the combination of DETA NONOate with polymyxin B causes indifferent effects.

5.2.3 The Checkerboard test to investigate synergy of NO with antibiotics.

A checkerboard method was conducted to determine whether the combination of DETA NONOate and antibiotics caused synergistic, indifferent, additive, or antagonistic effects. This experiment was carried out in 96-well plates. Defined minimal medium was inoculated (3%) with an overnight starter of *E. coli* EC958. Then, 200 μl of culture was added into each well. Freshly prepared stock solutions of antibiotics and DETA NONOate were used and the correct dilution of each

compound was added to each well. Every row has serial dilutions of either one compound or the combination of the two compounds (see section 2.7 in chapter 2). The 96-well plate was incubated in a plate reader at 37 ºC, 200 rpm for 24 h.

From the OD_{600} readings after 24 h incubation, the MIC and the combined MIC were determined and the combined effects were analyzed by a fractional inhibitory concentration (FIC) index. FIC was calculated as the MIC of antibiotic and DETA NONOate in combination, divided by the MIC of the antibiotic or DETA NONOate alone, Then, the FIC index was determined by adding the FICs. The following formula interprets the ΣFIC:

$$FIC_{\text{compound 1}} = \frac{MIC_{\text{compound 1}} \text{ in combination}}{MIC_{\text{compound 1}} \text{ alone}}$$

$$FIC_{\text{compound 2}} = \frac{MIC_{\text{compound 2}} \text{ in combination}}{MIC_{\text{compound 2}} \text{ alone}}$$

$$\Sigma FIC = FIC_{\text{compound 1}} + FIC_{\text{compound 2}}$$

When ΣFIC is ≤ 0.5, this indicates a synergistic effect. This means that the activity of two compounds in combination is greater than the sum of their individual effects. If ΣFIC > 4 the two compounds show antagonistic effect which means that the activity of the two compounds in combination is less than the sum of their individual effect. If 0.5 < ΣFIC ≤ 4 the two compounds show indifferent or additive effect, for example, the activity of the two compounds in combination is equal to the sum of their indivisiual effects (Chung et al., 2011). Table 5.1 shows the MICs of the antibiotics alone or in combination with DETA NONOate and it also shows the fractional inhibitory concentrations (FICs) for studying compound interaction with bacteria. The MIC of cefotaxime alone was 200 μg/ml and the MIC of cefotaxime in combination with DETA NONOate was 100 μg/ml while the MIC of DETA NONOate alone was 1 mM and in combination with cefotaxime, the MIC was 0.2 mM. The ΣFIC was 0.7 less than 4 and more than 0.5.

This finding indicates that adding DETA NONOate causes an additive or indifferent effect on the activity of cefotaxime.

The MIC of doxycycline alone was 50 μg/ml and it was 100 μg/ml when doxycycline was combined with DETA NONOate. The MIC of DETA NONOate alone was 1 mM and it was 2.5 mM when it was combined with doxycycline and the ΣFIC was 4.5 (Table 5.1). This result demonstrates that DETA NONOate apparently antagonised the activity of doxycycline, in agreement with the results in Fig. 5.3 C.

Additive or indifferent effects were observed in combinations of DETA NONOate with polymyxin B. The MIC of polymyxin B alone was 2 μg/ml and it was also 2 μg/ml when doxycycline was combined with DETA NONOate. The MIC of DETA NONOate alone was 1 mM and it was 1 mM when DETA NONOate combined with antibiotics. Thus, The ΣFIC was 2. This finding indicates that adding DETA NONOate combined with polymyxin B did not cause any change in the activity of the antibiotics. The MIC of gentamicin alone was 1.6 μg/ml and it was 1 μg/ml when gentamicin was combined with DETA NONOate, while the MIC of DETA NONOate alone was 1 mM and it was 0.2 mM when it combined with gentamicin. The ΣFIC was 0.8 (Table 5.1). This result also shows another additive effect caused by the combination of DETA NONOate with antibiotics. In conclusion, it seems like DETA NONOate does not have any synergistic effects when combined with the tested antibiotics. However, it shows antagonistic effect when it is combined with doxycycline. By understanding the interaction of the antibiotic with the targets inside bacterial cells, it may help us to explain the antagonistic effects that doxycycline and DETA NONOate cause when combined.

Table 5.1 Minimum Inhibitory Concentrations (MICs) of cefotaxime, doxycycline, polymyxin B and gentamicin alone or in Combination with DETA NONOate and fractional inhibitory concentrations (FICs) for antimicrobial resistant *E. coli* EC958.

Antibiotics	$MIC_{antibiotics}$ alone (μg/ml)	$MIC_{antibiotics}$ in combination with DETA NONOate (μg/ml)	$MIC_{DETA\ NONOate}$ alone (mM)	$MIC_{DETA\ NONOate}$ in combination with antibiotics (mM)	ΣFIC	Interaction
Cefotaxime	200	100	1	0.2	0.7	Additive/ indifference
Doxycycline	50	100	1	2.5	4.5	Antagonistic
Polymyxin B	2	2	1	1	2	Additive/ indifference
Gentamicin	1.6	1	1	0.2	0.8	Additive/ indifference

5.2.4 The minimum inhibitory concentrations of doxycycline on *E. coli* MG1655 and the *hmp* mutant strains

Based on the antagonistic effect between doxycycline and DETA NONOate, the *E. coli* MG1655 and the *hmp* strains were tested to investigate whether the activity of doxycycline might be impaired by Hmp. We reasoned that if NO directly antagonises the antibacterial action of doxycycline, then removing Hmp and elevating intracellular NO levels would further antagonise the action of the antibiotics (see Fig. 5.4)

The minimum inhibitory concentrations (MICs) of doxycycline on *E. coli* wild type and *hmp* strains were determined by the broth dilution test as in section 5.2.1. DETA NONOate was tested at concentrations 0.1-2.5 mM for wild type and at 0.1-1 mM for the *hmp* mutant strain. Doxycycline was tested at concentrations 3-50 μg/ml for wild type and at 0.8-50 μg/ml for the *hmp* strain (Fig. 5.5).

Figure 5.5 and 5.6 showed the growth curve of *E. coli* wild type and the *hmp* strains with different concentrations of doxycycline and DETA NONOate. Doxycycline (3 μg/ml) caused a prolonged lag in the *E. coli* wild type growth lasting 10 h, then cells started recovery, while 6, 12.5, 25 and 50 μg/ml doxycycline prevented the growth of *E. coli* wild type (Fig, 5.5 A). The MIC was 6 μg/ml of doxycycline against the wild type strain. A significant reduction in bacterial growth occurred when *E. coli* wild type was treated with 0.2 mM of DETA NONOate lasting a few hours, then cells started to recover. DETA NONOate (0.3 mM) also caused a significant reduction in the growth of the wild type lasting 16 h, followed by cells recovery (Fig. 5.5 B). Cells treated with 0.6, 1 and 2.5 mM DETA NONOate stopped the growth of the wild type strain and the MIC was 0.6 mM.

On the other hand, treating the *hmp* strain with 0.8 μg/ml doxycycline caused slight reduction in the cells growth while 1.6 μg/ml doxycycline inhibited the growth for 5 h before the cells started to recover. Upon addition of 3 μg/ml doxycycline, a prolonged lag in growth lasting ~ 10 h was observed prior to cell recovery whereas 6, 12.5, 25 and 50 μg/ml doxycycline prevented the *hmp* strain growth (Fig, 5.6 A). Therefore, the MIC was 6 μg/ml of doxycycline against the *hmp* strain.

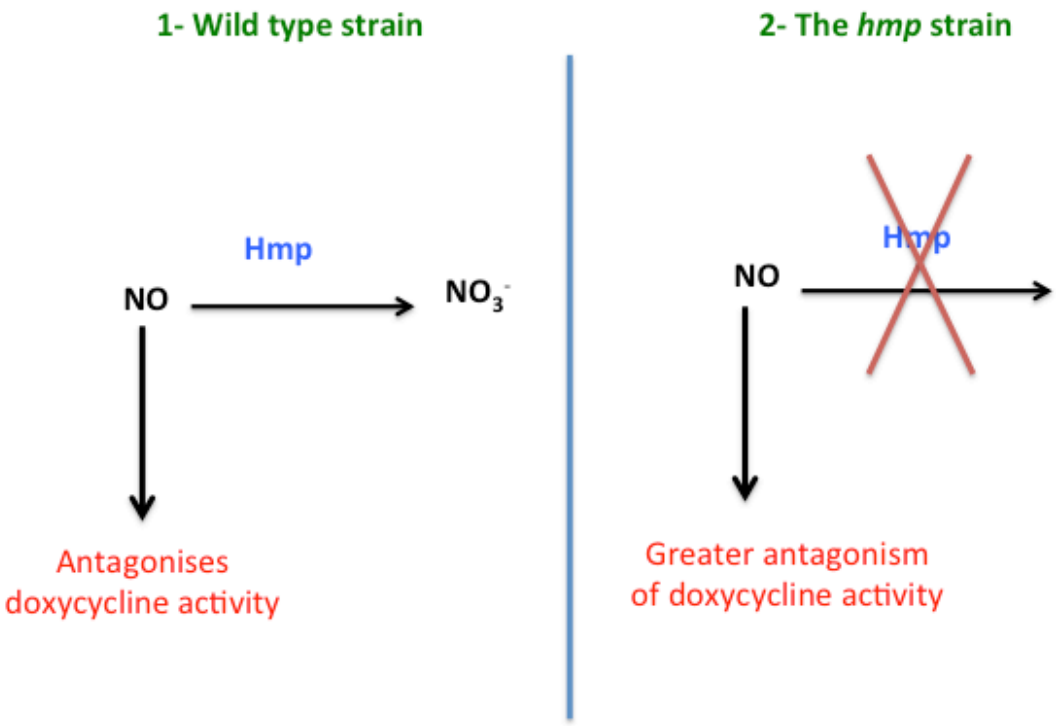


Figure 5.4 Flow chart describing the hypothesis of using the *hmp* strain in order to investigate the role that Hmp play in doxycycline activity

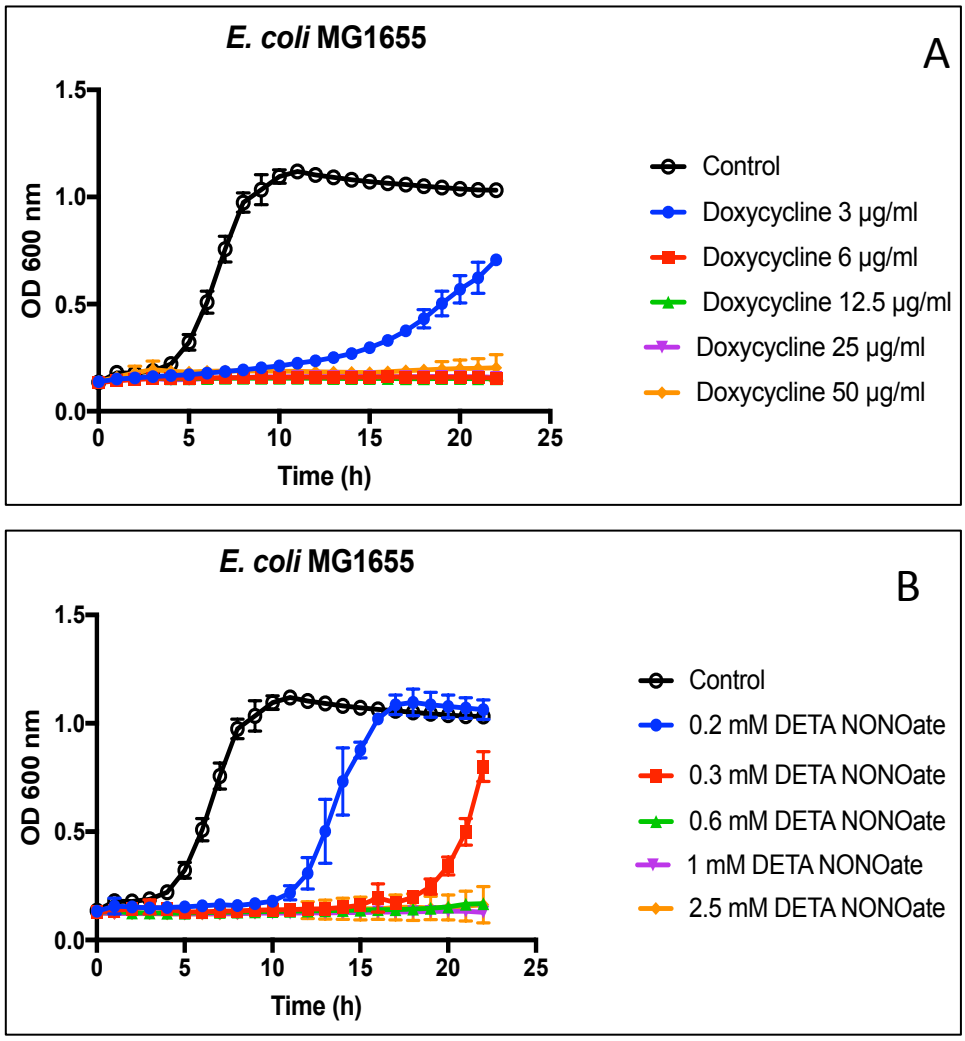


Figure 5.5 The minimum inhibitory concentrations of antibiotics and DETA NONOate for *E.coli* MG1655

The *E. coli* MG1655 strain was grown in defined minimal medium for 24 h at 37°C, 200 rpm and OD_{600} was recorded every hour. Doxycycline and DETA NONOate were added at time zero (A) The effect of different concentrations of doxycycline 3, 6, 12.5, 25 and 50 µg/ml on the growth of *E. coli* MG1655. (B) Effects of different concentrations of DETA NONOate 0.2, 0.3, 0.6, 1 and 2.5 mM on the growth of *E. coli* MG1655. Control experiments were carried out in the absence of antimicrobial agents. Data presented are the mean of 3 biological repeats ± S.E

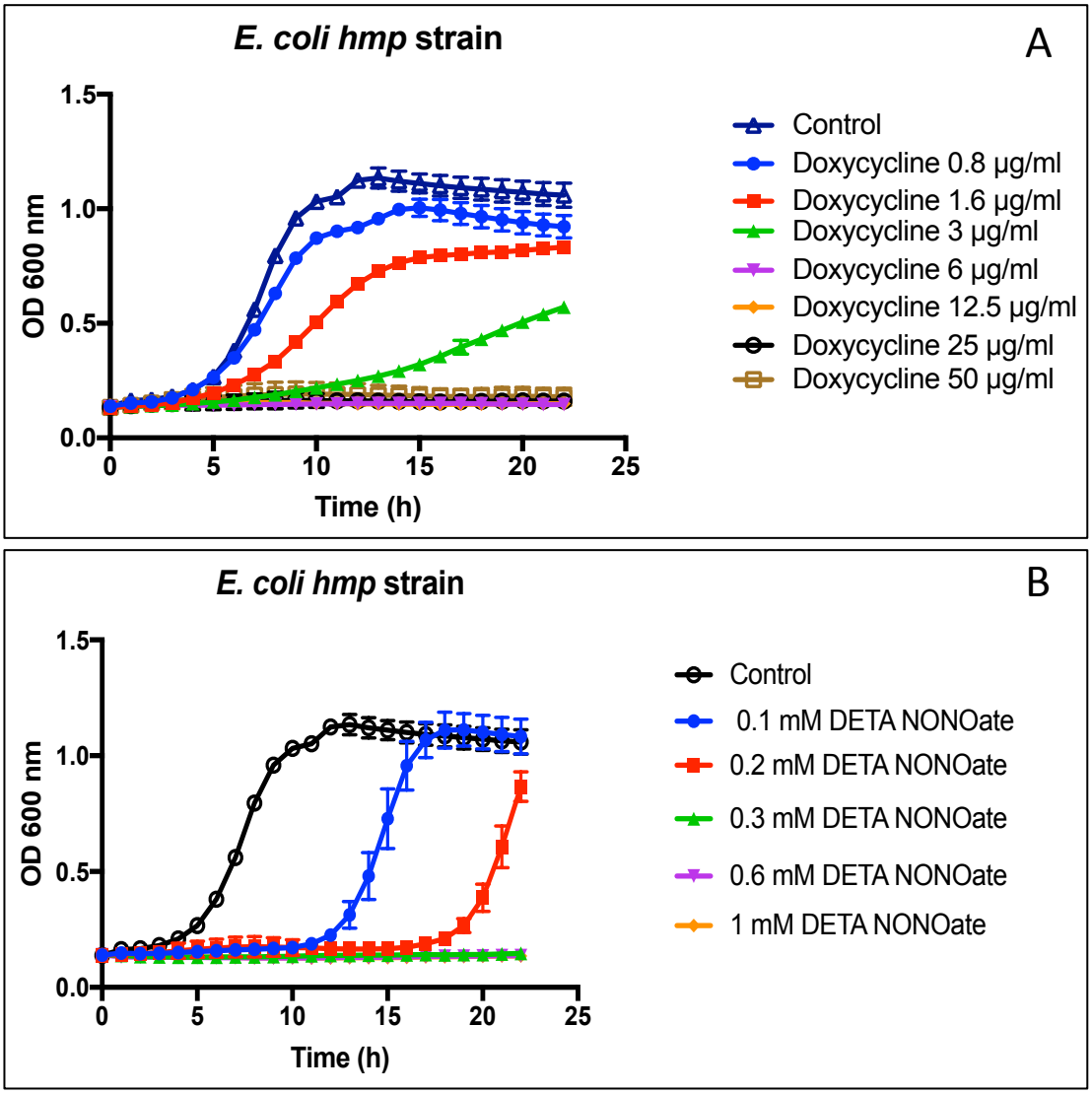


Figure 5.6 The minimum inhibitory concentrations of antibiotics and DETA NONOate for *E.coli hmp* strain

E. coli hmp strain was grown in defined minimal medium for 24 h at 37°C, 200 rpm and OD_{600} was recorded every hour. Doxycycline and DETA NONOate were added at time zero (A) The effect of different concentrations of doxycycline 0.8, 1.6, 3, 6, 12.5, 25 and 50 µg/ml on the growth of *E. coli hmp* strain. (B) Effects of different concentrations of DETA NONOate 0.1, 0.2, 0.3, 0.6 and 1 mM on the growth of *E. coli hmp* strain. Control experiments were carried out in the absence of antimicrobial agents. Data presented are the mean of 3 biological repeats ± S.E

DETA NONOate (0.1 mM) caused a significant reduction in the growth of *hmp* strain and 0.2 mM DETA NONOate was also caused a significant reduction in the growth lasting for 16 h prior to cell recovery.

Treating the cells with 0.3, 0.6 and 1 mM DETA NONOate prevented the growth of *hmp* strain. Thus, the MIC was 0.3 mM (Fig.5.6 B). The *hmp* strain was more sensitive to DETA NONOate than wild type (Figures 5.5 and 5.6) and this result was expected due to the lack of Hmp in this strain which has a main role in detoxifying NO.

5.2.5 The effect of NO on the MIC of Doxycycline against *E. coli* MG1655, *hmp* mutant and EC958

In order to investigate the interacting effects of DETA NONOate combined with doxycycline against *E. coli* MG1655 and *hmp* mutant strains, the checkerboard method was conducted as in section 5.2.3 to determine the MICs and the combined MICs. The combined effects were analyzed by a fractional inhibitory concentration (FIC) index.

Based on the FIC calculations (Table 5.2), the combinations of doxycycline and DETA NONOate caused antagonistic effects for wild type and *hmp* strains with FIC indices of 5.9 and 6.2 respectively. Table 5.2 shows the MIC of the doxycycline alone against *E. coli* MG1655 and *hmp* stains was 6 µg/ml and it was increased to 25 µg/ml in combination with DETA NONOate. For *E. coli* MG1655, the MIC of DETA NONOate alone was 0.6 mM and it was also increased to 1 mM when it was combined with doxycycline while 0.3 mM DETA NONOate concentration alone was the MIC for *hmp* strain and it was 0.6 mM when it combined with doxycycline. As mentioned previously, DETA NONOate showed antagonistic effects for *E. coli* EC958. Figure 5.7 A and B shows the growth curve of *E. coli* MG1655 and *hmp* strains when cultures were treated with 6 µg/ml of doxycycline with different concentrations of DETA NONOate. The doxycycline concentration used in this growth curve the MIC. When bacteria were treated with the doxycycline alone, no growth was observed through 24 h incubation in both wild type and *hmp* mutant strain.

Table 5.2 Minimum Inhibitory concentrations (MICs) of doxycycline alone or in Combination with DETA NONOate and fractional inhibitory concentrations (FICs) for MG1655, *hmp* mutant and EC958 strains. The data for EC958 were also shown in Table 5.1.

Strain	MIC doxycycline alone (μg/ml)	MIC doxycycline in combination with DETA NONOate (μg/ml)	MIC DETA NONOate alone (mM)	MIC DETA NONOate in combination with doxycycline (mM)	ΣFIC	Interaction
E. coli MG1655	6	25	0.6	1	5.9	Antagonistic
E. coli hmp mutant	6	25	0.3	0.6	6.2	Antagonistic
E. coli EC958	50	100	1	2.5	4.5	Antagonistic

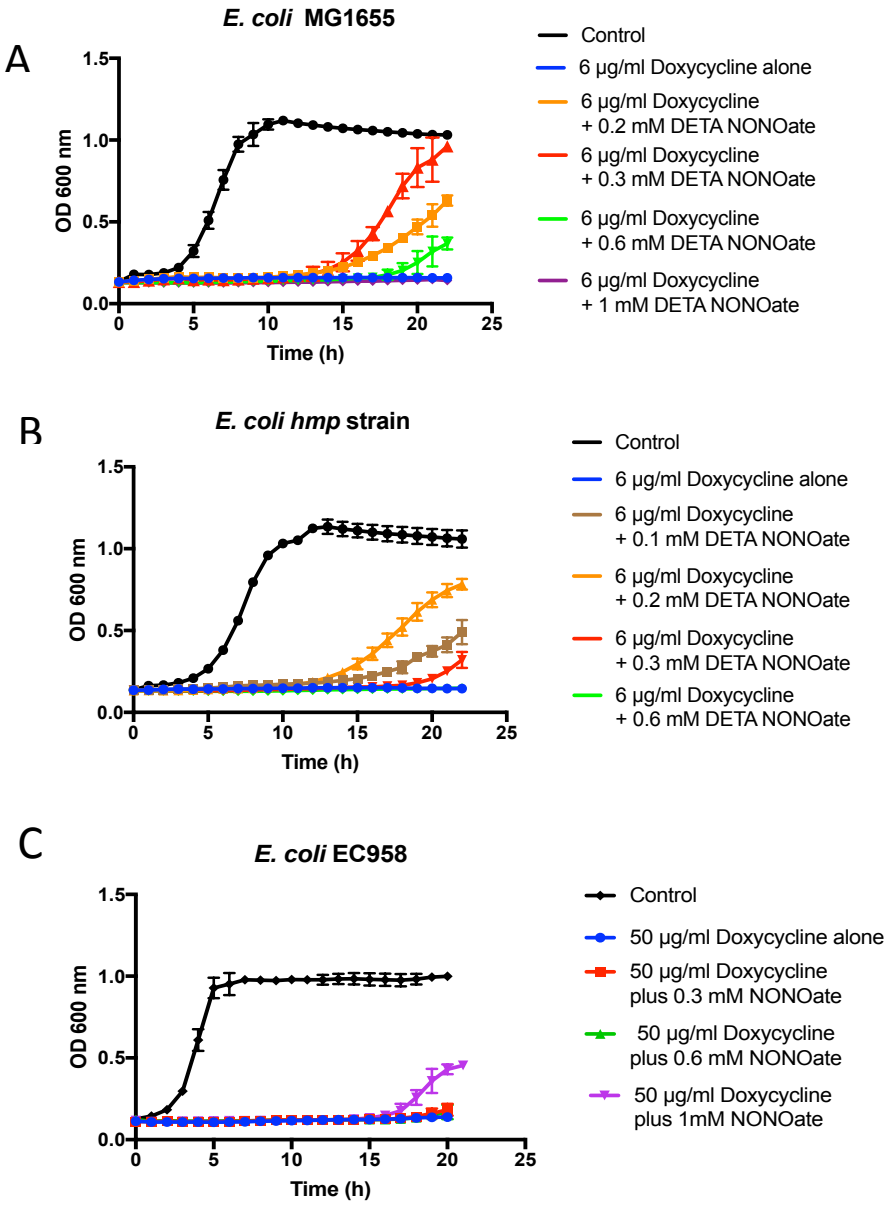


Figure 5.7 The effect of different concentrations of DETA NONoate on the MIC of doxycycline

E. coli MG1655, *hmp* mutant and EC958 strains were grown in defined minimal medium for 24 h at 37°C, 200 rpm and OD_{600} was recorded every hour. The MIC concentrations of doxycycline was added individually or combined with different concentrations of DETA NONOate. (A) *E. coli* MG1655. (B) *E. coli hmp* strain. (C) *E. coli* EC958. Control experiments were carried out in the absence of antimicrobial agents. Data presented are the mean of 3 biological repeats ± S.E

On the other hand, adding 0.2 mM of DETA NONOate to 6 μg/ml of doxycycline caused a significant reduction at the beginning of the incubation. After 14 h incubation, *E. coli* MG1655 started to recover. This result suggests that NO protects bacterial cells from the harmful effects of the doxycycline. When bacterial cells were exposed to 0.6 mM of DETA NONoate plus 6 μg/ml of doxycycline, bacterial cells recovered to a lesser extent compared to exposing the cells to 0.2 mM and 0.3 mM of DETA NONOate plus 6 μg/ml of doxycycline (Fig. 5.7 A). The explanation of this result might be related to the fact that treating wild type cells with 0.6 mM DETA NONOate alone caused an inhibition in the growth. Thus, fewer bacterial cells are available to recover from these high concentrations of both DETA NONOate and doxycycline.

Adding 0.1 mM DETA NONOate to the *hmp* mutant strain was enough to protect bacterial cells from the toxic effect of doxycycline 6 μg/ml. Moreover, increasing the DETA NONOate concentration (to 0.2 mM) elicited an increase in the ability of *hmp* strain to recover from high concentration of doxycycline (Fig. 5.7 B). This result corresponds to the wild type result and the variation between the two strains is related to the sensitivity of *hmp* strain to the high concentration of DETA NONOate.

As mentioned previously, *E. coli* EC958 was more resistant to DETA NONOate and doxycycline and showed antagonistic effects. However, the antagonistic effect of these two compounds clearly occurred when the bacterial cells were exposed to high concentrations of DETA NONOate compared to the *E. coli* MG1655 and *hmp* strains. This result might be related to *E. coli* EC958 being more resistant to DETA NONOate and doxycycline compared to the wild type and *hmp* strains. Therefore, a high concentration of DETA NONOate might be needed to protect the bacterial cells from high concentration of doxycycline.

In conclusion, doxycycline alone is effective against *E. coli* MG1655, *hmp* strain and EC958. However, adding DETA NONOate in combination with doxycycline can make the doxycycline less effective. The explanation for this result is not clear but NO may affect doxycycline uptake.

5.3 Discussion

The aim of the work in this chapter was to investigate if DETA NONOate has antibacterial effects against multidrug-resistant *E. coli* EC958 with a special focus on the combination effects of DETA NONOate and antibiotics. *E. coli* EC958 is one of the major pathogenic bacteria which cause a urinary tract infection (UTI). This strain produces the extended–spectrum β lactamase (ESBL). Moreover, it is fluoroquinolone resistant and belongs to the *E. coli* ST131 group (Forde et al., 2014). Due to the multidrug resistant characteristics that this strain has, and the critical clinical condition that it causes, the combination of more than one antimicrobial agent might be the best treatment option to impair the spread of this multidrug resistant bacterial infection (Lai et al., 2016).

It is clear from the results obtained that *E. coli* EC958 is most resistant to cefotaxime of the antibiotics tested as shown in Figure 5.1 A. This is due to *E. coli.* EC958 having genes encoding extended–spectrum β lactamase (ESBL) which makes the strain more resistant to third generation cephalosporins such as cefotaxime (Phan et al., 2015). Figure 5.3 A shows that 0.2 mM DETA NONOate plus 100 μg/ml of cefotaxime can inhibit EC958 growth. However, the checkerboard test result demonstrates that DETA NONOate in combination with cefotaxime showed additive but not synergistic effects against EC958. Previous work demonstrates that NO can modulate β-lactam antibiotic activity and induce antibiotic tolerance to *E. coli*, nontyphoidal *Salmonella enterica serovar Typhimurium* and *B. pseudomallei* (Jones-Carson et al., 2014). Jones-Carson et al. (2014) showed that NO induces resistance of β-lactam antibiotics to *B. pseudomallei* and other Gram-negative bacteria by repression of the electron transport chain.

When bacterial cells were treated with gentamicin plus DETA NONOate, additive effects were observed according to checkerboard test results (Table 5.1). Previous studies suggest that NO can promote the resistance of bacterial cells to aminoglycoside antibiotics by inhibiting the energy–dependent phases of aminoglycoside uptake (McCollister et al., 2011).

Polymyxin B alone affected growth of *E.coli*. EC958 (Fig.5.1 D). No significant changes in the antibiotic effect was observed when NO was added to bacterial culture

in combination with polymyxin B (Fig.5.3 D).

The bactericidal activity of tetracycline depends on the interaction of the antibiotic with the 30S subunit of bacterial ribosomes (Noah et al., 1999, Chopra and Roberts, 2001). Bacteria can utilize several mechanisms to induce the resistance to tetracycline such as efflux of the antibiotic, ribosome protection and modification of antibiotic (Chopra and Roberts, 2001). Doxycycline shows a wide spectrum of activity against several pathogens, including Gram-negative bacteria (Zhanel et al., 2004).

Lai et al. (2016) demonstrated the activity of doxycycline against multidrug-resistant *E. coli* and found that many of the clinical isolates that they tested, including ESBL, were not sensitive to doxycycline. However, when they combined even sub-inhibitory concentrations of an aminoglycoside with doxycycline, this combination showed synergistic activities against more than 80% of the clinical isolates that they tested. In addition, they found that the combination of doxycycline with other drugs can enhance the doxycycline activity and can manage the multidrug-resistant *E. coli* infections.

Adding DETA NONOate to doxycycline shows antagonistic activity against EC958 with an ΣFIC of 4.5. This suggests that NO protects the bacteria from doxycycline. Gusarov et al. (2009) found that treating a *B. subtilis nos* mutant strain (lack of bNOS) with acriflavine (ACR) or pyocyanin (PYO) caused a significant reduction in bacterial growth compared with the wild-type strain. This confirm that NO reduced the toxicity of the antimicrobial agent that they used. They also demonstrated that NO impaired ACR activity via two mechanism; (i) ACR has an arylamino group which reacts with NO^+ and changes the chemistry of the compounds to be less toxic. (ii) NO reduce the oxidative stress generated by ACR (Gusarov et al., 2009). In the case of pyocyanin, NO did not show any chemical reaction with PYO to make the compound less toxic. However, PYO induced superoxide formation during the stationary phase of *B. subtilis* which cause bacterial killing by ROS and NO protected bacteria by impaired ROS toxicity (Gusarov et al., 2009).

NO, NO_3^- and NO_2^- modulate the antimicrobial activity of different classes of antibiotics that target ribosomes (Vazquez-Torres and Baumler, 2016). The *hmp* mutant strain was tested due to the role that flavohemoglobin (Hmp) plays in

protection of bacteria against nitrosative stress. Lack of Hmp leads to an increase in the concentration of NO in the culture and bacterial cells became more sensitive to NO stress. Svensson *et al.* (2010) demonstrated that Hmp has a major role in protecting uropathogenic *E. coli* (UPEC) against NO. Moreover, a mouse infection model illustrated that UPEC *hmp* mutant colonization was less compared to the wild type strain. Figure 5.2 shows that EC958 is more resistant to DETA NONOate alone compared to MG1655 and *hmp* strains (Fig. 5.5 B and 5.6 B) and this correspond to the Svensson *et al.* (2006) study.

Checkerboard test results confirm that NO with doxycycline has antagonistic effects against *E. coli* MG1655 and *hmp* strains (Table 5.2) and have approximately the same response to these two compounds in combination. Comparing this result with the effect of DETA NONOate and doxycycline against EC958 strain can show the differences between these strains and how EC958 is even more resistant to the two compounds. It also confirms that DETA NONOate reduces the activity of doxycycline. It was hypothesized that NO works as an antioxidant to reduce the toxicity of doxycycline and make bacterial cells more resistant as showed in Figure 5.7. However, the tetracycline family is bacteriostatic and these drugs do not produce hydroxyl radicals (Kohanski et al., 2007). It is likely that NO blocks the drug uptake as demonstrated in McCollister et al. (2011), which explains how NO can protect bacteria from aminoglycosides by nitrosylating the terminal quinol cytochrome oxidase which is important for uptake of the drug. On other hand, Coban and Durupinar (2003) showed that the combination of NO with ciprofloxacin, pefloxacin and ofloxacin against *S. typhimurium* clinical isolates showed an antagonistic effect. However, combining these stresses against a *soxRS* mutant and *acr* mutant strains showed a synergistic effect and that was related to NO triggers genes that are involved in antibiotic resistance.

Chapter 6
General discussion

Chapter 6 General discussion

The innate immune system in the mammalian host responds to pathogens by generating nitrosative and oxidative stresses that include O_2^-, H_2O_2, HOCl and NO (Fang, 2004). The synthesis of these sets off a cascade of subsequent reactions leading, for example, to $ONOO^-$, NO^+ and NO^-. ROS (O_2^-, H_2O_2, HOCl) and RNS (NO and its redox products) have major roles in microbial infection (Fang, 2004) Phagocytic cells such as macrophages kill internalised bacteria by a number of mechanisms including an initial (6-12 h) 'respiratory' burst of reactive oxygen species (ROS) generated by the phagocyte oxidase phox. This is followed by production of NO from inducible NO synthase (iNOS), leading to various RNS, leading in turn to sustained bacteriostasis at 24-48 h (Slauch, 2011, Vazquez-Torres et al., 2000, Robinson, 2008). Unsurprisingly, bacteria have evolved tightly regulated adaptive responses to RNS and ROS.

For example, *E. coli* and *S. enterica* Typhimurium use two major mechanisms to detoxify NO (Poole, 2005a), namely the flavohaemoglobin Hmp and the flavorubredoxin NorV. The former catalyses primarily an O_2-dependent denitrosylase ('dioxygenase') reaction converting NO to nitrate ion, or, with lower activity, the anoxic reduction to NO^- (nitroxyl) and N_2O (Kim et al., 1999, Poole and Hughes, 2000) Regulation of Hmp expression is achieved by integrating the activities of several transcription factors (TFs) that sense NO *per se* (Fnr, NsrR) or indirectly by the consequences of NO exposure on thiol biochemistry (MetR) and Fe pools (Fur) (Membrillo-Hernández et al., 1998, Hernandez-Urzua et al., 2006). Tight control of Hmp expression is essential because of the propensity of the protein to generate ROS in the absence of NO (Gilberthorpe et al., 2007). Flavorubredoxin NorV with its cognate reductase, NorW catalyses the reductive detoxification of NO to NO^- under microaerobic/anaerobic conditions (Gardner et al., 2002). The *norVW* operon is regulated by NorR (Spiro and D'Autreaux, 2012). The bacterial defence mechanisms deployed upon exposure to ROS may detoxify the ROS (e.g. superoxide dismutase, catalase, peroxidase), repair the damage caused by ROS (e.g. iron-sulfur cluster biosynthesis), replace vulnerable components (e.g. synthesis of fumarase C), or protect cell components from damage (e.g. iron sequestration and DNA shielding by ferritin and Dps) various superoxide dismutases (Storz and Imlay, APR 1999). In

enteric bacteria these responses are coordinated by two transcription factors; (i) OxyR, which responds to peroxide and nitrosative stress (Marshall et al., 2000) to activate expression of approximately 20 operons (in *E. coli*) and (ii) SoxR, which responds to redox-cycling drugs and NO to control a regulon of approximately 25 operons (in *E. coli*) that encode proteins with anti-oxidant roles.

In addition, to the above well-established mechanisms of protection against NO and related species, *E. coli* has several other enzymic strategies to resist RNS. These are not covered in this thesis but are briefly described here. *E. coli* contains the diiron protein YtfE to repair iron-sulfur clusters damaged by nitrosative stress (Vine et al., 2010), and also possesses the NO-inducible cytochrome *bd*-I respiratory oxidase that confers resistance to NO (Mason et al., 2009, Pullan et al., 2007). Finally, efflux of glutathione and cysteine by the ABC transporter CydDC has also been shown to provide tolerance to NO (Holyoake et al., 2016). The cytochrome *bd*-I complex, encoded by the *cydAB* operon, is expressed maximally in microaerobic environments and is up-regulated in response to NO (Pullan et al., 2007). Rather than catalyzing the decomposition of NO, cytochrome *bd*-I is an NO-tolerant terminal oxidase of the respiratory chain that permits aerobic respiration in the presence of NO and low oxygen.

In addition, new evidence has emerged on the hybrid cluster protein, Hcp, which contains a 4Fe-2O-2S iron-sulfur-oxygen cluster, unique in (van den Berg et al., 2000). Hcp is required for NO resistance in several bacteria but its role in enterobacteria has remained obscure. In *Salmonella enterica* Typhimurium, Hcp has been implicated in resistance to nitrite, pathogenicity and in NO detoxification, but the mechanism is unclear (Karlinsey et al., 2012). In *E. coli*, increased sensitivity of an *hcp* mutant to *S*-nitrosoglutathione and macrophage-derived NO has been observed (Filenko et al., 2007). The two-gene operon, *hcp-hcr* is expressed only during anaerobic growth and was not studied in this work. However, the fact that it requires FNR for expression, and is repressed by the NO-sensitive repressor, NsrR, as is *hmp* that we study here (Constantinidou et al., 2006) makes it an interesting candidate for extending the present work to anoxic or low-oxygen conditions. Although a role for Hcp in protection against nitrosative stress has been widely predicted, only recently did Wang et al. (2016) demonstrate that Hcp is a high affinity

nitric oxide (NO) reductase that, in the absence of the reductase NorVW, is essential for survival under low, physiologically relevant conditions of nitrosative stress (Wang et al., 2016). Deletion of *hcp* results in extreme sensitivity to NO during anaerobic growth and inactivation of the iron-sulfur proteins, aconitase and fumarase.

In general, studies have been demonstrated the transcriptional responses of *E. coli* to a number of (but not all) the agents that bacteria encounter in the macrophage environment. These are GSNO (Flatley et al., 2005) and NO (Pullan et al., 2007) under both aerobic and anaerobic conditions, peroxynitrite (McLean et al., 2010b), hydrogen peroxide (Zheng et al., 2001, McLean et al., 2010b) and hypochlorous acid (Gray et al., 2013b). The main conclusion is that most of these stresses are distinct and non-overlapping. For example, although GSNO, NO and peroxynitrite ($ONOO^-$) are called RNS, their biological effects are distinct. The clear distinction, for example, between the effects of GSNO and NO is in accord with the chemistry of these species: GSNO is a nitrosating agent, NO *per se* is not. $ONOO^-$ responses do not overlap with those for GSNO but $ONOO^-$ and NO each elicit mechanisms for repairing iron-sulfur clusters. Indeed, study the transcriptional responses of combined nitrosative and oxidative stress was demonstrated for limited number of bacteria (Nobre and Saraiva, 2013).

In this thesis, the effect of combining three stresses (NO, H_2O_2 and HOCl) has been tested. In chapter 3, recovery studies revealed that *E. coli* cells survive and recover from various nitrosative and oxidative stresses. In addition, an *hmp* mutant strain has approximately the same behavior as the wild-type in both the recovery and stress phases. It is therefore clear that Hmp is not only the defence that *E. coli* needs to survive and recover from these stresses and this is consistent with the diversity of NO RNS protective measures surveyed above. Although Hmp plays the major role in protecting bacterial cells from nitrosative stress in macrophages (Stevanin et al., 2002, Baptista et al., 2012, Shepherd et al., 2016), one study demonstrated that macrophages do not completely eliminate *Salmonella* and viable *S. enterica* can still be recovered 21 h after co-incubation with murine macrophages even at relatively low initial multiplicities of infection and after prolonged priming with IFN-g (Gilberthorpe et al., 2007). Thus, bacteria cells that survive and recover from

macrophage/neutrophil assaults must have adapted to a number of hostile environmental factors that include at least nitrosative and oxidative stress.

Future work could include dissecting in much greater detail the transcriptomic and cellular responses that occur when cells are introduced into, or removed from, media containing stress reagents, when a process of adaptation must occur. Such detailed studies at a transcriptomic level have been reported for the survival of *Salmonella* within macrophages or on infection of epithelial cells (Eriksson et al., 2003, Hautefort et al., 2008) and for *E. coli* readapting to growth at different oxygen concentrations (Rolfe et al., 2011). Such a study could also employ proteomics to monitor changes in intercellular proteins and also a detailed systems analysis to identify the key transcription factors (Rolfe et al., 2012). Furthermore, as proposed above, it would also be interesting to study gene expression under microaerobic/anaerobic conditions.

In chapter 4 is reported for the first time the effects of three stresses (NO, H_2O_2, HOCl) on the expression of genes responsive to nitrosative and oxidative stress and the effect of sequential exposure of these stresses. This revealed that oxidative stress-related genes such as *katG, ahpC* and *sodA* were up-regulated in response to the stresses in combination or when sequential exposure of two stresses was applied. Thus, these genes are required for *E. coli* to survive from the combined effect of the three stresses or when cells are treated with one stress first then followed with the other. An earlier study Eriksson et al. (2003) compared the transcriptional profile of intracellular *Salmonella* with the whole genome expression profile of *E. coli in vitro* as described in (Zheng et al., 2001). They found that only 6 oxidative related genes were up-regulated in intracellular *Salmonella* compared to 30 genes were up-regulated in *E. coli*. The gene expression profile suggested that bacteria were exposed to some peroxide stress and the OxyR response, as evidenced by expression of *trxC* that encodes thioredoxin-2. Notably, *hmp* expression is induced at 8 h, coinciding with NO synthesis by iNOS, as well as *norRVW* responsible for anoxic or microaerobic NO detoxification (Eriksson et al., 2003).

In the present study the effects of combined stress on the expression of nitrosative stress response genes were tested and the study showed that *hmp* gene was highly up-

regulated in response to combined stresses. This confirm the important role that *hmp* play in bacterial resistance to macrophages stresses (Stevanin et al., 2002, Stevanin et al., 2007, Baptista et al., 2012, Shepherd et al., 2016). On the other hand, the expression of *norV* were affected when *E. coli* were exposed to combined three stress or two stresses such as NO plus H_2O_2 or NO plus HOCl. This result confirmed that H_2O_2 and HOCl impaired the expression of *norV* when cells were exposed to combining stresses. Baptista et al. (2012) study demonstrated the role of H_2O_2 in blocking the expression of *norV*. Moreover, in contrast with the role of *hmp* to protect bacterial cells at all stage within macrophage infection, *norV* has time-limited role within macrophages infection. The transcriptional profile after macrophage infection in Eriksson et al. (2003) study showed that the expression of *norV* was highly up-regulated following the nitrosative stress burst. In order to investigate the effects of combined oxidative and nitrosative stresses on HOCl response gene, the *nemA* gene was tested and the study revealed that the gene was up-regulated in response to HOCl alone, NO alone, NO and H_2O_2 or NO and HOCl. Besides the role that NemA plays in detoxification of NEM and reactive electrophiles, it also has a role in degradation of toxic nitrous (Umezawa et al., 2008). This confirms the up-regulation of *nemA* in response to NO. Although NO is a highly reactive radical and it could react with HOCl or H_2O_2, NO electrode experiments were conducted in order to investigate whether HOCl or H_2O_2 might change NO chemistry and stability in solution. NO electrode data revealed that neither HOCl nor H_2O_2 affected NO chemistry. In future work, due to the up-regulation of *katG* expression observed in response to each individual stress and the combined stresses, it may be useful to validate these finding by measuring catalase activity of cells under the same condition. Furthermore, biochemical studies are also required to understand whether NemA has any role in detoxifying NO.

In chapter 5, NO did not show any synergistic effect against EC958 strain when it was combined with the selected antibiotics. Indeed, when NO was combined with doxycycline, it showed antagonistic effects against EC958, MG1655 and *hmp* mutant strains. The antimicrobial activity of doxycycline relies on the ability of the antibiotic to react with the 30S subunit of bacterial ribosomes (Noah et al., 1999, Chopra and Roberts, 2001). NO can alter the antimicrobial activity of different classes of antibiotics that target ribosomes (Vazquez-Torres and Baumler, 2016), and it has a

role in changing the toxic activity of antibiotics against gram-positive or gram-negative bacteria (Gusarov et al., 2009, McCollister et al., 2011). Many studies have demonstrated that NO works as an antioxidant to reduce the toxicity of the antibiotics that generated ROS in order to kill bacteria (Gusarov et al., 2009). However, NO can nitrosylate the terminal quinol cytochrome oxidase which is important for uptake of the drug (Vazquez-Torres and Baumler, 2016). Future work should focus on the effect of NO on doxycycline uptake by *E. coli* and study the chemical interactions that could occur when NO is combined with doxycycline *in vitro*.

In this work, the data for each result was presented as a mean of 3 biological repeats ± S.E. However, for the results that showed small differences between the categories, the statistical test ANOVA was applied to test whether these small differences were statistically significant. For example, in section 5.2.2, a small difference was found between the effects of DETA NONOate in combination with doxycycline on bacterial growth and the effect of doxycycline alone. The use of the ANOVA statistical test confirmed that there was a significant difference between these two conditions and this was further confirmed by using a checkerboard test.

Finally, although this work has used one important pathogenic strain of *E. coli* – the uropathogen EC958 – future studies should focus on other relevant strains. *Escherichia coli* is responsible for common, serious infections not only in humans but also animals. This is of great concern since it is suggested that farmers need to dramatically cut the amount of antibiotics used in agriculture, because of the threat to human health. The concern in agricultural antibiotic use is driving up levels of antibiotic resistance, leading to new "superbugs" (http://www.nhs.uk/news/2015/12December/Pages/Antibiotic-use-in-farm-animals-threatens-human-health.aspx). This report looked at resistance to antimicrobial drugs, which includes antibiotics as well as antifungal and antiparasitic drugs. Resistance to these drugs is collectively known as antimicrobial resistance (AMR).

E. coli infections are responsible for significant economic losses in the poultry industry worldwide and *E. coli* is the aetiological agent responsible for avian colibacillosis, a complex respiratory and systemic disease that causes substantial welfare and economic costs in this industry worldwide (La Ragione et al., 2013, La

Ragione and Woodward, 2002). Losses are incurred through mortality, condemnation of carcasses at slaughter, reduced productivity and costs associated with vaccination and antibiotic treatment. Recent epidemiological evidence suggests that approximately 39% of mortalities from broiler flocks are associated with colibacillosis (Kemmett et al., 2014). Avian colibacillosis is a multifactorial disease and a number of risk factors are known, including prior or concurrent infection with respiratory viruses or Mycoplasma, stress and injury associated with formation of a social hierarchy, onset of sexual maturity and intense laying, and poor biosecurity, hygiene and ventilation.

Detailed analysis of Avian Pathogenic *E. coli* (APEC) genome sequences has revealed a number of similarities with *E. coli* normally associated with human extra-intestinal infections, such as the UPEC (EC958) used in this study and ascending urinary tract infections, sepsis and neonatal meningitis (Mellata et al., 2012). Indeed poultry have recently been demonstrated to be reservoirs for *E. coli* O45 serotypes implicated in human infections such as meningitis (Mora et al., 2013). Knowledge of specific virulence determinants and genes and their involvement in septicaemic disease is limited, but several virulence determinants have been characterised including genes for adhesins (La Ragione et al., 2000) and toxins. As in all *E. coli* strains, there is an apparent redundancy of systems for transporting iron as Fe(II), haem or via siderophores. Recently, salmochelin (a C-glycosylated form of enterobactin) and aerobactin have been shown to be especially important in a chicken challenge model of APEC (Gao et al., 2012)

In the poultry industry, antimicrobial agents are usually administered at the flock level, rather than treating individual birds. Thus, ensuring accurate dosing for all birds is challenging and can result in increased risk of selection of antibiotic-resistant bacteria. Antimicrobial combinations may exert synergistic effects and minimise the risks of drug resistance, toxicity and inactivation of antibiotics by other bacteria (Abu-Basha et al., 2012). Diseased poultry flocks frequently require treatment with antibiotics, and resistance to first line antibiotics is increasingly common and of further concern is the emergence of multi-drug resistant *E. coli* strains in poultry, including those encoding extended spectrum beta-lactamases (ESBLs), cephalosporin-resistance and plasmid-mediated quinolone resistance (Overdevest et

al., 2011). Furthermore, from an economic perspective, there is a need to avoid the use of medicines that have extended withdrawal times. Moreover, the concerns regarding antimicrobial resistance are compounded by evidence of direct transmission of APECs to humans (Ojeniyi, 1989) and the emergence of new strains. There is therefore an urgent need for alternatives to antibiotics, such as the leading live-attenuated APEC vaccine (Poulvac *E. coli* (La Ragione et al., 2013)). It would be interesting to test the hypothesis that combinations of antimicrobial agents, such as those used in the present thesis, might contribute to the control of farm-related bacterial pathogens.

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