Regulation and Sources of Nitric Oxide in *Escherichia coli*

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Abstract

Enteric bacteria such as *Escherichia coli* live in an oxygen-limited environment where they are exposed to NO essentially from three sources: NO generated by host defence mechanisms, by other bacteria or as a product of their own metabolism. In response to exposure to NO, various transcription factors regulate the expression of genes that provide protection against nitrosative stress, but their respective roles remain controversial. This thesis has focused on three of these transcription factors, OxyR, NsrR and FNR. Key questions to be answered were whether S-nitrosylated OxyR directly regulates the expression of NO-regulated genes; whether NsrR is required for the synthesis of an important protective system; and whether FNR is a physiologically relevant sensor of environmental NO. Deletion of *oxyR* had no effect on the activation of transcription of the *hcp* promoter in response to anaerobic growth in the presence of nitrate or nitrite, or during aerobic growth. The activity of the *hcp* promoter in an fnr^+ strain was much higher than in an *fnr* mutant, suggesting that the effects of an *oxyR* mutation on resistance to nitrosative stress are indirect rather than direct, while FNR is essential for *hcp* expression. An *nsrR* deletion was neither beneficial nor detrimental for growth under nitrosative stress. Therefore, NsrR is not essential for the synthesis of a key protection mechanism or one that depends upon repair of NsrR by the hybrid cluster protein, Hcp. Nitric oxide produced during nitrite reduction had no effect on the ability of FNR to activate or repress expression of *hcp::lacZ* and *hmp::lacZ* fusion promoters, respectively. However, both of these promoters were derepressed due to inactivation of NsrR, not FNR. This confirmed that any effect of FNR on expression of NsrR-regulated genes is due to chemical damage at concentrations of NO far higher than those required to derepress expression of genes regulated by NsrR. Nitric oxide is generated by bacteria as a side product

during nitrate reduction via nitrite to ammonia. The nitrate reductase, NarG, is the major source of NO generation, with the two nitrite reductases, NirBD and NrfAB serving as secondary sources. This NO is reduced to ammonia by NrfAB, or to N_2O by NorVW and Hmp. Isogenic strains designated JCB52XX were constructed based upon a quadruple mutant that lacks genes for all four of these activities, Nir, Nrf, NorV and Hmp. The *hcp* gene encoding a high affinity NO reductase and *narG* were also deleted from some of these strains. The current work demonstrates that some NO accumulates in the *E. coli* cytoplasm even in this quadruple mutant and that the source of most of this residual NO is the NsrR-regulated protein, YtfE. Sensitivity of the *ytfE*$^+$ strain to NO was completely complemented by transformation with the *norV*$^+$ plasmid. The ability to resist even low levels of nitrosative stress depended critically on a functional Hcp-Hcr system. Further deletion of the *ytfE* gene reversed this dependence upon Hcp-Hcr. Transcription of *hcp* promoter in the *napAB narG ytfE* strain was lower than that in the *napAB narG ytfE*$^+$ strain. The presence of the pET-24a::*ytfE*$^+$ plasmid had almost no effect on growth of the *ytfE*$^+$ host, JCB5270, in the presence or absence of nitrite, but suppressed growth of the *ytfE*, JCB5280, transformant. It is possible that YtfE repairs proteins damaged by nitrosative stress by releasing NO from nitrosylated iron-sulfur proteins, and that Hcp reduces these low levels of NO to the less toxic N_2O. This proposal is contrary to that proposed by other groups that YtfE repairs iron-sulfur centres by replacing iron atoms released during nitrosative stress.

Dedication

Dedicated with love and thanks, my wonderful husband Maher, my kids Allen,Ava and to my family

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List of Abbreviations

Amp	Ampicillin
APS	Ammonium persulphate
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
Carb	Carbenicillin
Cat	Chloramphenicol acetyl transferase
Chlor	Chloramphenicol
CIAP	Calf intestinal alkaline phosphatase
C-	Carboxyl terminal
DAN	2,3-diaminonapthalene
DMK	Demethylmenaquinone
DMS(O)	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNIC	Di-nitrosyl iron complex
dNTP	Deoxynucloside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
GSNO	S-nitrosoglutathione
Hcp	Hybrid cluster protein
iNOS	(inducible) Nitric oxide synthase
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
Kan	Kanamycin
kb	Kilo base

LB	Lennox broth
LB-Ca2+	Lennox broth supplemented with 2 mM CaCl2
M	Molar
MGD	Molybdopterin guanine dinucleotide
MK	Menaquinone
MPT	Molybdopterin
MS	Minimal salts
MS	Minimal salts medium
NA	Nutrient agar
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (redused form)
NADP(H)	Nicotinamide adenine dinucleotide phosphate
ND	Not determined
NO	Nitric oxide
NOSW	Nitric oxide saturated water
N-	Amino terminal
OD	Optical density
ONPG	Orthonitrophenyl-β-D-galactopyranoside
PCR	Polymerase chain reaction
RIC	Repair of iron centres
RNAP	RNA polymerase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSNO	S-nitrosothiols
SNO-Cys	S-nitrosocysteine

TBE	Tris-borate-ethylene diamine tetra acetic acid
TCA	Tricarboxylic acid
Tet	Tetracycline
TMA(O)	Trimethylamine (*N*-oxide)
Tris	Tris (hydroxymethyl) aminomethane
UQ	Ubiquinone
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER ONE -INTRODUCTION

1.1 The enteric bacterium *Escherichia coli* and its adaptation to anaerobic growth

Escherichia coli is a Gram negative and facultatively anaerobic enterobacterium that has been found in different environments including the mammalian gut (Drasar *et al*., 1966). It has various metabolic abilities to adapt to environments with differing oxygen levels such as aerobiosis (outside a host) or anaerobosis (inside the host) as a part of its life cycle. Hence, oxygen plays a vital role in the survival of this organism (Cole, 1996; Ingledew and Poole, 1984). As a facultatively anaerobic bacterium, *E. coli* is able to switch from aerobic to anaerobic respiration once oxygen is absent. Therefore, *E. coli* can utilise diverse alternative electron acceptors in order to respire efficiently (Unden *et al*., 1995), which include nitrate, nitrite, fumarate, trimethylamine N-oxide (TMAO), and dimethyl sulphoxide (DMSO) (Unden and Bongaerts, 1997).

Nitrate and nitrite are energetically the two most powerful oxidants present in the mammalian gastro-intestinal tract and *E. coli* can readily reduce them to ammonia (Cole and Brown, 1980; Cole, 1987; Cole 1996; Saul *et al.* 1981). In the gastro-intestinal tract, this organism must be able to protect itself against host defence mechanisms. These include nitrogen-based chemicals that destroy the bacterium. In addition, *E. coli* and other microorganisms that share the gastro-intestinal tract generate toxic intermediates during nitrate reduction (Bogdan *et al*., 2000; Nathan, 1997). It has been shown that *E. coli* contains several methods for reducing or detoxifying nitric oxide (Poole *et al.,* 1996; Tucker *et al*., 2005; Browning *et al*., 2006).

The di-iron protein, YtfE, has been extensively implicated in the repair of iron-sulfur centres of proteins damaged by nitrosation (Justino *et al.*, 2006; 2007; 2009; Norbe *et al.*, 2014; Overton *et al.*, 2008). The aims of the current study were to discover more precisely the biochemical function of YtfE in protecting bacteria against nitrosative stress and its regulation the response of *E. coli* to the same conditions. Therefore, in this chapter the role of nitrate reduction in the nitrogen cycle will be described. The main transcription factors that are involved in anaerobic conditions will be introduced followed by a description of the enzymes that reduce nitrate and nitrite. Also in this chapter the generation of reactive nitrogen species, particularly the formation of nitric oxide (NO) and the damage caused by NO will be described. The mechanisms that detoxify NO, which includes HmpA, NrfA, and NorVW will be introduced. This report will cover the main sensors of nitrosative and oxidative stress, NsrR and OxyR, which detect NO and hydrogen peroxide respectively in the *E. coli* cytoplasm. Finally, the HCP protein that might be involved in protection against nitrosative stress will be detailed, and the aims of this study will be described.

1.2 Bacterial nitrogen cycle

Nitrogen is one of the fundamental basic elements for all living organisms. It is a significant component of the two pivotal biological macromolecules, proteins and nucleic acids. Processing of nitrogen from one form to another composes the global biogeochemical nitrogen cycle (Moreno-Vivian *et al.*, 1999). Some bacterial species such as *Klebsiella pneumonia* and *Azotobacter vinelandii* have nitrogenase enzymes, which in turn use these enzymes to assimilate di-nitrogen gas (N_2) into ammonia by process called nitrogen fixation. Denitrification is an anaerobic reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and nitrogen by denitrifying bacteria (Figure 1.1) (Berks *et*

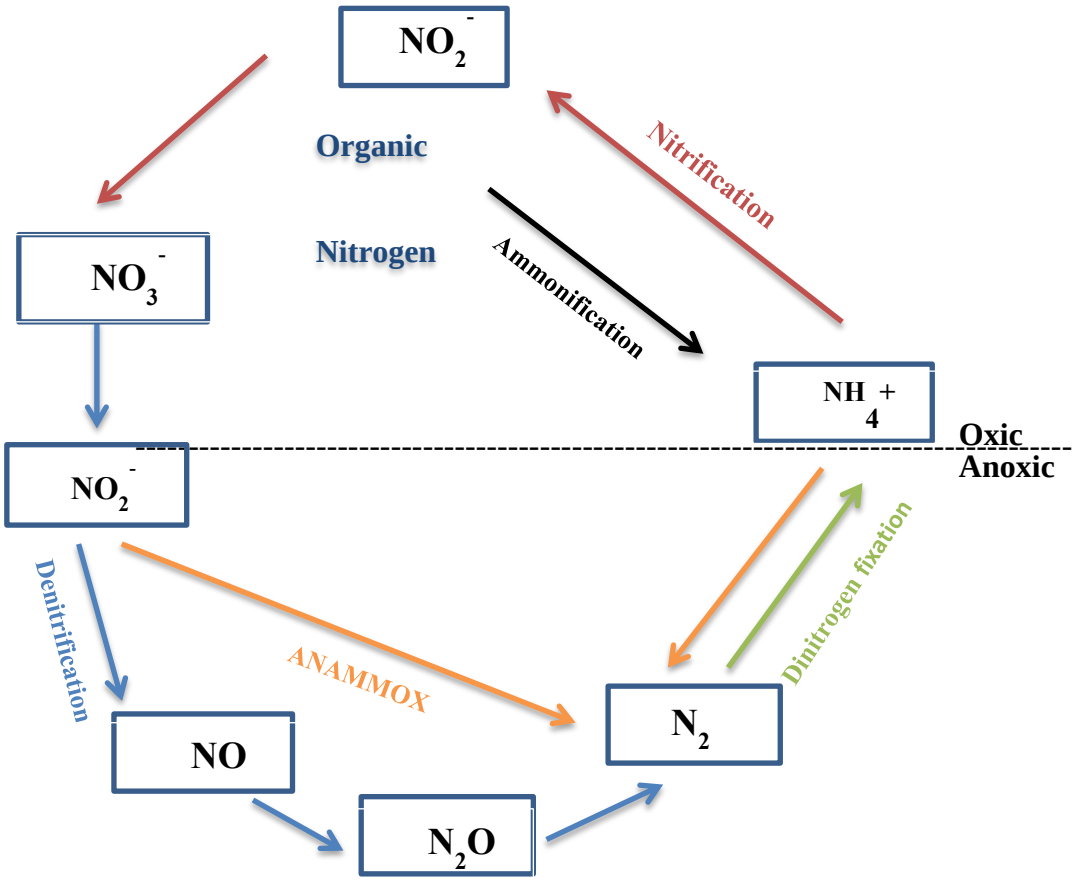


Figure 1.1: The bacterial nitrogen cycle.

Denitrification consists of series of reactions that include respiratory reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and (N_2) (coloured blue). The first part of nitrification is the oxidation of ammonia (NH_4^+) to nitrite and nitrate (coloured red). The conversion of organic matter into ammonium by bacteria or fungi called Ammonification (coloured black). The conversion of atmospheric nitrogen to ammonium constitute dinitrogen fixation (coloured green). The direct conversion of nitrite and ammonium into nitrogen gas (N_2) is known as ANAMMOX (coloured orange).

al., 1995a; Ferguson, 1998). Nitrification is the oxidation of ammonium (NH_4^+) to nitrite and then to nitrate through the activity of two nitrifying bacteria including *Nitrozomonas europaea* (ammonium oxidizer) and *Nitrobacter volgaris* (nitrite oxidizer).

Ammonification is the conversion of organic matter by bacteria or fungi to ammonium. Moreover, the anaerobic conversion of combined ammonium and nitrite by Planctomycetes directly into dinitrogen and water is known as anammox (an abbreviation of ANaerobic AMMonium OXidation) (Figure 1.1) (Jetten *et al*., 2001; Jetten *et al*., 2005). The nitrate reduction process plays a critical role in the nitrogen cycle and has considerable influence in different ways, including agricultural activities and public health (Cole and Brown, 1980).

The intensive use of fertilizers in agriculture causes accumulation of high levels of nitrate in groundwater, and this can cause many issues for supplies of drinking water and is linked to gastric cancer (Butler, 2003; Lane, 2007). In addition, N_2O that is produced from denitrification is a potent green-house gas that has a damaging impact on the ozone layer. However, nitrate respiration is a critical biological process that occurs in anoxic conditions, by which nitrate is a terminal electron acceptor for the generation of metabolic energy (Richardson *et al*., 1998; Moreno-Vivian *et al*., 1999). Nitrate reduction forms a key role in the biogeochemical nitrogen cycle, and hence, nitrate reduction by bacteria has been a topic of interest for researchers over many years.

1.3 Anaerobic respiration processes in *E. coli*

During anaerobic growth, *E. coli* conserves energy from the transfer of electrons from various electron donors to electron acceptors other than oxygen including nitrate, nitrite ,TMAO, DMSO and fumarate. This process leads to the reduction of alternative electron

acceptors, particularly nitrate via specific nitrate reductases (Klein *et al.*, 2006; Richardson, 2000; Unden and Bongaerts, 1997). Thus, a trans-membrane proton electrochemical gradient or proton-motive force (pmf) is a consequence of the free energy generated from the electron transfer process. Then the released energy is used by the cell to produce ATP through the utilization of membrane-bound ATP synthase (Ferguson, 1998; Richardson, 2000). Figure 1.2 illustrates the generation of proton-motive force (pmf), which couple with electron transfer. The anaerobic respiratory system in *E. coli* or electron transport chain consists of several primary electron donating dehydrogenases that transfer electrons to the final electron accepting reductases, which are linked by a pool of quinones as a widespread redox mediator.

The oxidation of these dehydrogenase enzymes provides electrons to the terminal anaerobic electron acceptors. These dehydrogenases comprise isoenzymes for some of the compounds that play a vital role as electron donors including formate, NADH, glycerol-3-phosphate, and hydrogen (Figure 1.3). Oxygen and nitrate compounds are used as electron acceptors. *E. coli* encodes a number of specific quinones, which are lipid soluble cofactors that function as mediators of electrons between dehydrogenases and terminal reductases (Unden and Bongaerts, 1997).

Quinones have hydrophobic properties that enable them to move freely between the reductants and oxidants in the electron transport chain (Soballe and Poole, 1999). These quinones consist of three types including ubiquinone or coenzyme Q (UQ); menaquinone or vitamin K2 (MQ); and demethylmenaquinone (DMQ) (Unden., 1988; Wallace and Young, 1977). The amount of these quinones produced is different depending upon the growth conditions (Bentley and Meganathan, 1987; Unden, 1988). It has been shown that the UQ cellular concentration is the same under aerobic and anaerobic conditions. However, higher

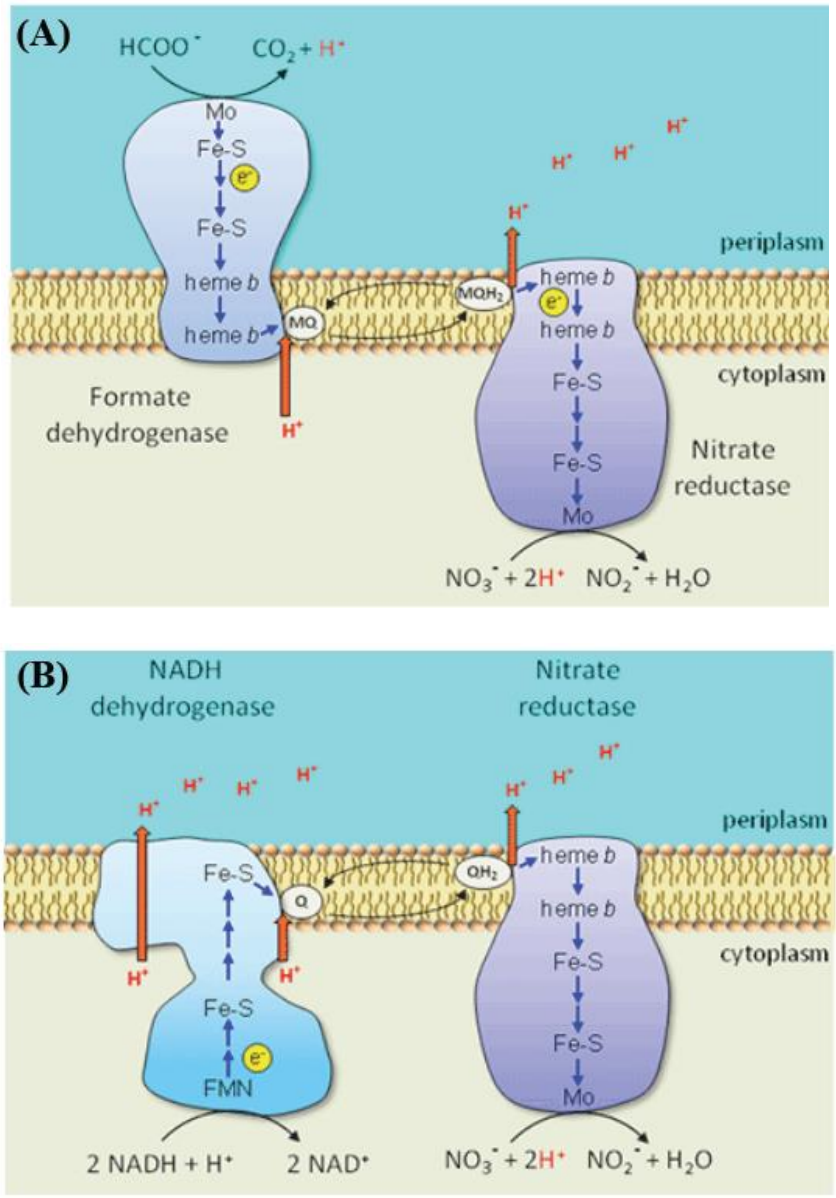


Figure 1.2: Bacterial electron transport chain.

Bacteria utilise formate (**A**) or NADH (**B**) as an electron donors. Nitrate is used as an electron acceptor (**A**), (**B**) for generation of pmf in order to produce ATP. These figures are taken from Gunsalus (2011).

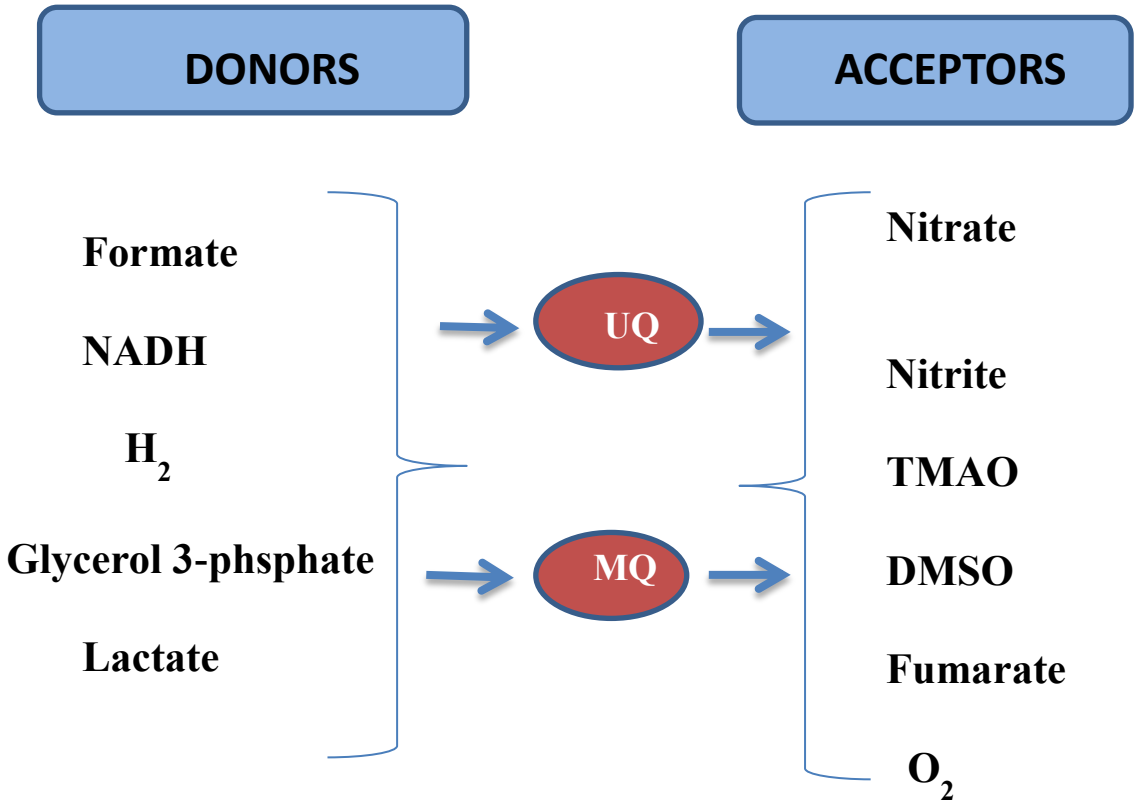


Figure 1.3: Electron donors and acceptors of *E. coli*.

The *E. coli* respiratory system is composed of a number of dehydrogenases that transfer electrons from electron donors to a pool of quinones like ubiquinone (UQ) and menaquinone (MK). UQ and MK are reoxidised by terminal oxidases that pass electrons to the final electron acceptors in order to generate ATP when oxygen is not available. Adapted from Gunsalus (2011).

amounts of MK and DMK are produced during anaerobic growth (Wissenbach *et al*., 1990). Brondijk *et al*. (2004) showed that UQ or MK donate electrons to NarG, while DMK and MK donate electrons to fumarate, TMAO and DMSO reductases (Unden and Bongaerts, 1997). A result of this biological process is the reduction of nitrate to nitrite and water. However, formation of a high level of nitrite is toxic to bacteria, therefore nitrite needs to be ejected out of the cytoplasm or metabolized rapidly after its production (Cole, 1996). In this case, the nitrate/nitrite transporters, NarK and NarU, in *E. coli* and many enteric bacteria are used to export nitrite from the cytoplasm (Clegg *et al*., 2002; Jia and Cole, 2005). *E. coli* can also protect itself against high amounts of nitrite via the periplasmic nitrite reductase, NrfA, which reduces nitrite to ammonia (Cole, 1996; Wang and Gunsalus, 2000; Poock *et al*., 2002). Furthermore, NADH-dependent nitrite reductase, NirB, in the cytoplasm has also the ability to reduce nitrite to ammonia (Jackson *et al*., 1981; Jackson *et al*., 1982; Cole, 1996; Vine *et al*., 2011).

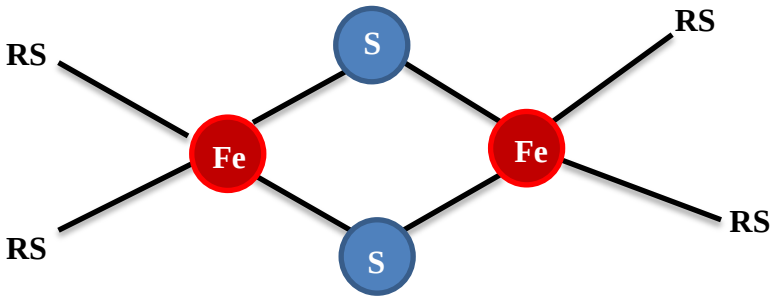
1.4 Iron-sulfur protein biogenesis and function

Iron-sulfur proteins were first discovered in the 1960s. These proteins are widespread in all forms of life (Beinert *et al*., 1997), contain Fe-S as a cofactor, which makes them ideal to sense, and respond to environmental signals (Mettert and Kiley, 2014). It has been shown that *E. coli* possesses nearly 80 iron-sulfur proteins that serve diverse functions in the cell (Py and Barras, 2010). Iron-sulfur clusters contain non-haem iron that couples with the sulfur atom in cysteine residues in the peptide chain, which in turn ligates to inorganic sulfur in the prosthetic group. Other amino acids can also act as an iron ligand, such as histidine, arginine, aspartate and serine (Py and Barras, 2010). Iron-sulfur clusters were characterised as vital cofactors of proteins involved in electron transport, enzymes and gene expression (Bian and

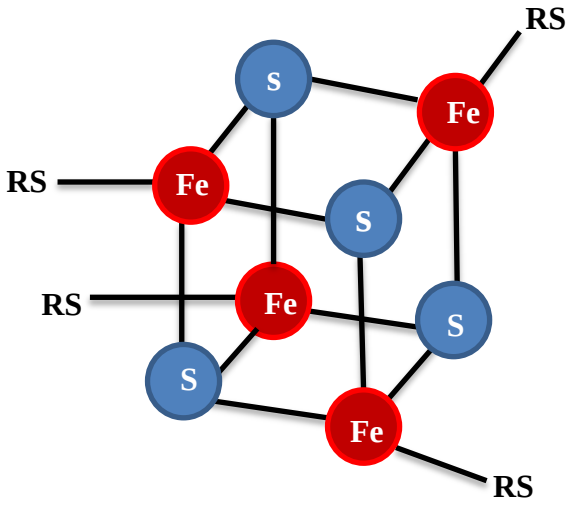
Cowan, 1999; Crack *et al*., 2014). There are various types of iron-sulfur clusters and two of them are most common including rhombic, [2Fe-2S] and cubic [4Fe-4S] structure (Figure 1.4). The *E. coli* iron cluster biogenesis has been extensively studied. So far, in all organisms three systems have been identified for Fe-S cluster biosynthesis, namely the Nif (the nitrogen fixation), the ISC (iron sulfur cluster) and the SUF (sulfur utilization factor) (Johanson *et al*., 2005; Barras *et al.*, 2005). Fe-S clusters of some regulatory proteins have unstable nature that leads to loss of activity when it is exposed to environmental stress such as O_2, NO and hydrogen peroxide (H_2O_2) (Beinert *et al*., 1997; Kiley and Beinert, 2003). Most proteins used in this study are iron-sulfur proteins such as FNR, NsrR, YtfE, Hcp and both aconitase and fumarase enzymes that will be discussed in detail in further sections.

1.5 The *E. coli* FNR global regulator of anaerobic respiration

In *E. coli*, the main regulator of anaerobic respiration is FNR, the regulator of fumarate and nitrate reduction, which regulates the adaptation of *E. coli* to anaerobic growth (Newman and Cole, 1978; Shaw and Guest, 1982; Jayaraman *et al*., 1988). This transcription factor is also able to repress the expression of genes implicated in aerobic metabolism, alongside its action as autorepressor of the *fnr* gene itself (Gunsalus and Park, 1994; Spiro and Guest, 1987; Spiro *et a*l., 1989). The iron sulfur centre [4Fe-4S] of FNR is used to sense whether oxygen is abundant or scarce (Green *et al.*, 1996; Khoroshilova *et al.*, 1997). During anaerobic growth, FNR contains a [4Fe-4S] cluster, which induces protein dimerization. This process provides an active form of FNR, which binds to a specific DNA site that closely matches a highly conserved sequence (TTGAT-N_4-ATCAA), thereby enhancing activation or



[2Fe-2S]



[4Fe-4S]

Figure 1.4: Structure of Fe-S clusters

The iron (red) of Fe-S is linked to inorganic sulfur in the prosthetic group (blue) and to cysteine residues in the peptide backbone (SR).

repression of transcription initiation by RNA polymerase (Jayaraman *et al*., 1988; Bell and Busby, 1994). In the presence of oxygen, the $[4Fe\text{-}4S]^{+2}$ clusters are converted to a $[2Fe\text{-}2S]^{+2}$ or $[3Fe\text{-}4S]^{+1}$ form that can dissociate into monomers that are unable to bind the DNA (Lazazzera *et al.,* 1996; Sutton *et al*., 2004a and 2004b). Hence, FNR is no longer able to control of transcription of specific genes in response to the presence or absence of oxygen (Fleischhacker and Kiley, 2011). A study by Cruz-Ramos *et al*. (2002) claimed that FNR can also respond to NO in addition to oxygen. As FNR is able to repress the transcription of gene that encodes NO detoxifying flavohaemoglobin, Hmp, and this repression can be overcome in the presence of NO. In this project, the physiological function of FNR will be investigated.

1.6 The *E. coli* two-component sensor-regulator proteins NarX and NarL

In addition to FNR, expression of genes under anaerobic conditions in response to nitrate and nitrite are also controlled by the two-component regulatory system, NarX-NarL (Darwin and Stewart, 1996; Stewart, 2003). NarX is a histidine sensor kinase that couples with its cognate cytoplasmic response regulator, NarL (Rabin and Stewart, 1993). The autophosphorylation of the sensor histidine kinase occurs at a conserved histidine in the presence of nitrate and nitrite, utilising ATP as a donor of a phosphate group (Figure 1.5). Then, the phosphate group can be transferred to the conserved aspartate residue in the response regulator, NarL (Stewart, 2003). Phosphorylated NarL is able to recognize specific DNA binding sites. Consequently, it activates or represses expression of different operons (Darwin and Stewart, 1996: Table 1.1) required for anaerobic respiration (Stewart, 1993, Stewart and Bledsoe, 2003, Constantinidou *et al.,* 2006). In *E. coli*, NarL co-ordinates with FNR to activate numerous genes involved in anaerobic respiration. In order to activate the nitrate reductase operon (*narGHJI*), FNR binds to the DNA at a site centred around -41.5,

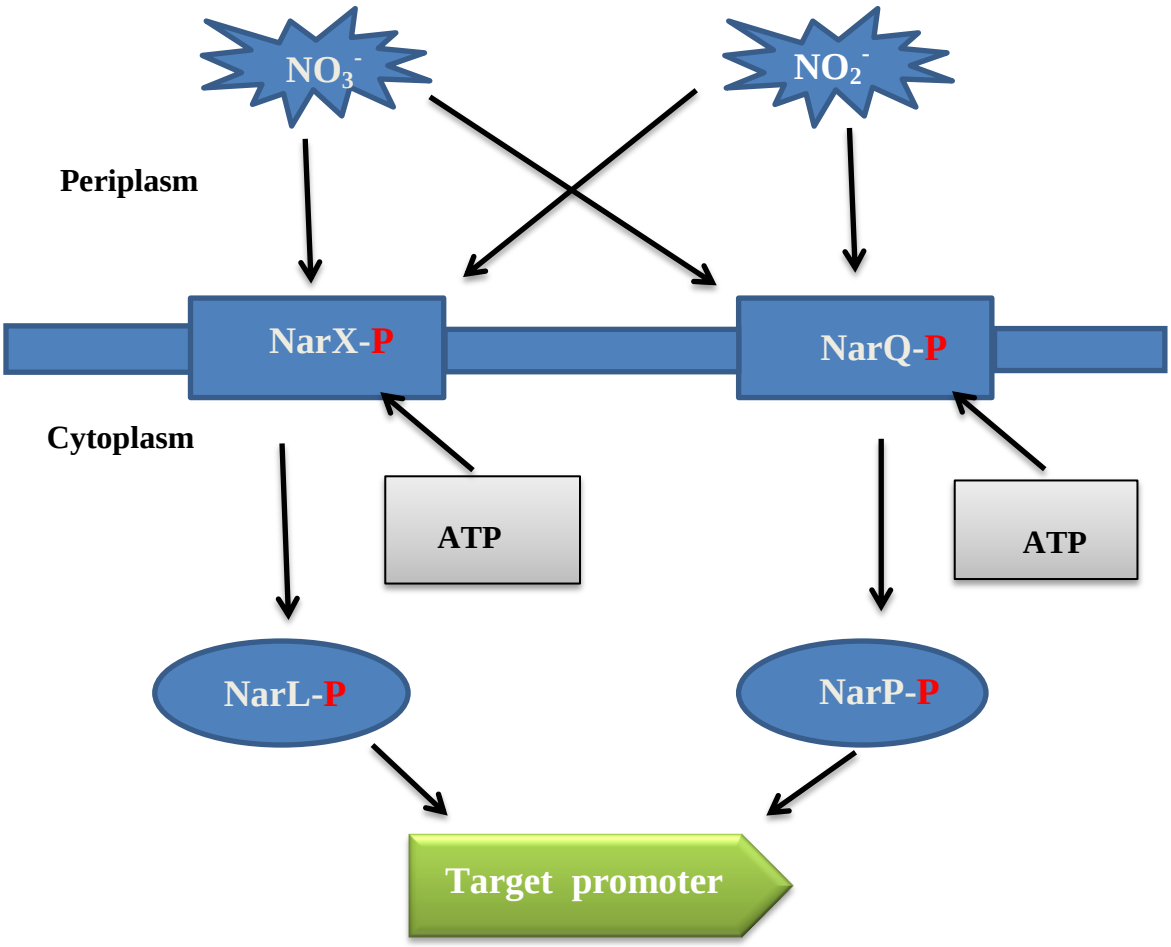


Figure 1.5: The two regulatory system NarXL and NarQP.

The dual-acting two-component regulators, NarXL and NarQP, activate and repress target promoters. In the presence of nitrate or nitrite in the periplasm causes the sensor components NarX and NarQ to autophosphorylate. The phosphate group is then passed through a phospho-relay system to the DNA target sequences, NarL and NarP, which in turn bind to the DNA binding components, specific promoter sequences, enhancing or repressing transcription. The sensitivity and response of NarX and NarQ to the different anions nitrate and nitrite differs allowing for differential control of target promoters dependent upon the environmental signal.

Table 1.1: *E. coli* NarL and NarP regulated locus

Key: **+**, positive activation, **-**, negative activation, **n/d**, not determined. Adapted from Darwin and Stewart (1996)

Enzyme	Operon	Effect of NarL	Effect of NarP
Nitrate reductase A	*narGHJI*	+	None
Periplasmic nitrate reductase	*napFDAGHBC*	-	+
NADH-dependent nitrite reductase	*nirBDC*	+	+
Cytoplasmic-*c* nitrite reductase	*nrfABCDEFG*	+/-	+
Nitrate/nitrite regulation	*narXL*	+	+
	narQ	-	None
	narP	-	None
Nitrite transport protein NarK	*narK*	+	None
Molybdate uptake	*modE*	+	n/d
DMSO reductase	*dmsABC*	-	n/d
Fumarate reductase	*frdABCD*	-	None
NADH dehydrogenase I	*nuoA-N*	+	+
Formate dehydrogenase N	*fdnGHI*	+	+

and NarL binds to single heptamers in the presence of high nitrate and low nitrite concentrations (Walker and DeMoss, 1991; Stewart, 1993; Li *et al*., 1994). , NarL also activates the expression of nitrate/nitrite transport protein (*narK*), and represses expression of fumarate reductase operon (*frdABCD*) and transcription of the (*dmsABC*) operon encoding DMSO reductase (Darwin and Stewart, 1996). Moreover, expression of periplasmic nitrate reductase, Nap, is totally dependent on FNR (Darwin and Stewart, 1995a; Grove *et al*., 1996) but its expression is repressed by phosphor-NarL in the presence of a high concentration of nitrate (Darwin *et al.,* 1998).

1.7 The *E. coli* two-component sensor-regulator proteins NarQ and NarP

NarQ is a membrane bound sensor that is phosphorylated in the same way as NarX in the presence of nitrate and nitrite (Figure 1.5). NarP is a response regulator that is participated in regulation of many operons (Darwin and Stewart, 1996, Table 1.1) that is critical for nitrite and nitrate respiration (Stewart, 1993, Stewart and Bledsoe, 2003; Noriega *et al.,* 2010; Constantindouin *et al*., 2006). In the presence of high nitrite concentrations, the induction of *nap* is totally dependent upon FNR and is then further activated by phospho-NarP, which binds to a 7-2-7 motif (Darwin and Stewart, 1995a; Grove *et al*., 1996). Some operons are activated by both Nar systems. For instance, the transcription of *nirB* operon is increased anaerobically in the presence of a high nitrate concentration by FNR binding to a site centred at position -41.5, and also requires binding of either NarL or NarP to a specific site centred around -69.5 (Jayaraman *et al*.,1989; Tyson *et al.,* 1993). During anaerobic growth, the expression of *nrf* operon that encodes formate–dependent nitrite reductase is activated in the presence of a limited concentration of nitrate and nitrite, as with other nitrate and nitrite reductases that previously discussed. The *nrf* promoter is activated in the presence of low

concentrations of nitrate and nitrite but it is repressed in the presence of high concentration of nitrate (Tyson *et al*., 1994; Wang and Gunsalus, 2000). The expression of this promoter is totally dependent on FNR bound to a site centred at -41.5 (Browning *et al*., 2005) and it is activated by NarL-P and NarP-P. However, when the nitrate concentration is high, the *nrf* promoter is repressed by NarL-P, thereby inhibiting transcription of the *nrf* gene (Tyson *et al*., 1994; Wang and Gunsalus, 2000). Other genes that are regulated by phospho-NarL and NarP (Table 1.1) include the formate dehydrogenase N *(fdnGHI*), NADH dehydrogenase I (*nuoA-N*) and the molybdate transporter *modABCD* (Darwin and Stewart, 1995b).

1.8 The bacterial nitrate reductases

There are three aims for reduction of nitrate in bacteria including (1) to obtain nitrate from the environment and to produce organic nitrogen compounds such as amino acids and nucleotides (nitrate assimilation); (2) the utilization of nitrate as a terminal electron acceptor for the generation of the electrochemical gradient (nitrate respiration); (3) decrease of excess reducing power for redox balancing (nitrate dissimilation). Various types of nitrate reductase perform the reduction of nitrate to nitrite. These enzymes are distinct in their structure, position in the cell, function and the final yield of the pathway in which they participate.

Bacteria contain three distinct types of nitrate reductase. In some bacteria, the nitrate reductase located in the cytoplasm is soluble, and utilizes ferredoxin, flavodoxin or NADH as the electron donor (Blasco *et al.*, 1990; Gangeswaran and Eady, 1996). This enzyme operates as a cytoplasmic assimilatory nitrate reductase (Nas) and uses nitrate as the source of nitrogen. The other two nitrate reductases are used for respiratory and dissimilatory tasks in order to generate metabolic energy and to disseminate increased reducing power for redox balancing (reviewed by Berks *et al*., 1995a; Moreno-Vivin *et al*., 1999; Richardson *et*

al., 2001). These enzymes include membrane-bound nitrate reductases (Nar), with the active site in the cytoplasm and the soluble periplasmic nitrate reductases (Nap) (Figure 1.6**)**. The active site of all three types of nitrate reductases is a molybdopterin cofactor, which is bound to guanosine monophosphate. Hence, it is described as the molybdoprotein guanine dinucleotide (MGD) (Rajagopalan and Johnson, 1992).

1.9 The molybdenum cofactor

The trace element molybdenum (Mo) is a significant micronutrient for many organisms such as bacteria, plants, and animals (Schwarz *et al*., 2009). Although these living organisms only need molybdenum in a small amount, the deficiency of this element is lethal for them (Stiefel, 2002). The *E. coli* chromosome has five genetic loci, *moa*, *moe*, *mob*, *mod,* and *mog*, which are responsible for production of functional molybdoenzyme. Deficiency in any of these genes leads to loss the activity of all molybdoenzymes (Rivers *et al*., 1993; Johann and Hinton, 1987; Nohno *et al*., 1988). It has been reported that more than 50 enzymes contain molybdenum and most of them were found in bacteria, while plants contain only four of them (Sigel and Sigel, 2002; Magalon *et al.*, 2011; Hille *et al*., 2011). Molybdenum is required for a broad range of redox active enzymes, which catalyse basic reactions in the metabolism of nitrogen, sulfur and carbon. In all organisms, molybdenum is inactive until it is complexed by a special compound in order to perform its catalytic role, and this compound is termed molybdopterin or metal containing-pterin (MPT). As a consequence of molybdenum incorporation into MPT, molybdenum cofactor (Moco) is generated for biological activity (Mendel, 2013). Apart from nitrogen-fixing nitrogenase, all molybdenum-containing enzymes harbour the pterin-type cofactor as the active compound for their catalytic site (Kisker *et al.*, 1997; Hille, 1996; Hu and Ribbe, 2013).

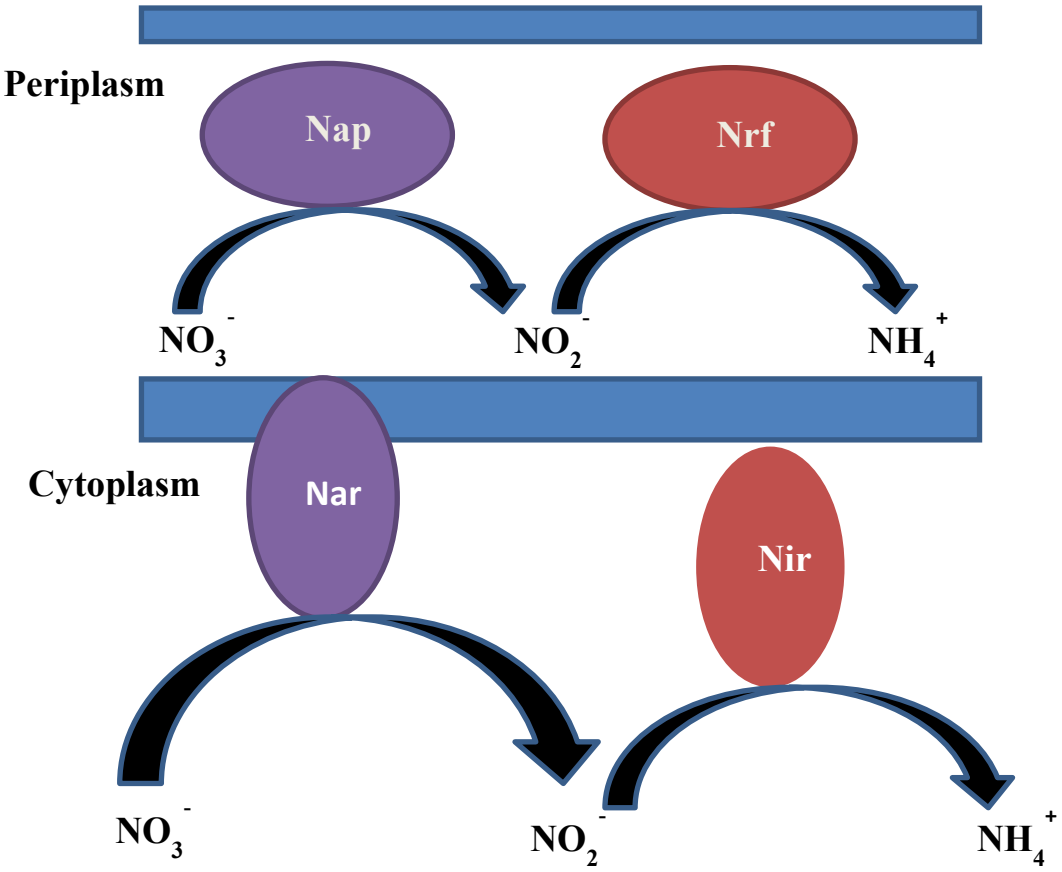


Figure 1.6*: E. coli* pathways for nitrate reduction to ammonium in the periplasm and cytoplasm

In this schematic diagram, *E. coli* has two pathways for nitrate ammonification via nitrite. The first periplasmic route is nitrate reductase Nap, encoded by *nap* operon, which reduces nitrate to nitrite in the periplasm, and thereby the generated nitrite is reduced by the c-type cytochrome nitrite reductases, NrfA. The second pathway includes nitrate reductase A encoded by *narGHJI* that reduces nitrate to ammonia in the cytoplasm in cooperation with the soluble cytoplasmic NADH dependent nitrite reductase, NirB.

It has been proposed that the dithiolene group of the 6-alkyl side chain of tricyclic pyranopterin (molybdopterin) is connected to the molybdenum atom in the molybdenum cofactor (Rajagopalan and Johnson, 1992). In addition, the sulphur atoms of the dithiolene group link to the molybdenum with a stoichiometry of one molybdopterin per molybdenum **(**Figure 1.7, C). Bacteria contain additional different molybdenum cofactors that are formed by the coordination of a second nucleotide, GMP, AMP, IMP and CMP to the phosphate group of molybdopterin (Rajagopalan and Johnson, 1992).

In *Rhodobacter sphaeroides,* the chemical nature and the basic structure of the bacterial MGD cofactor were first discovered in DMSO reductase (Johnson *et al*., 1990) (Figure 1.7, D). Molybdenum (Mo) and tungsten (W) have analogous chemical properties. In this case, the question is: is tungsten able to replace molybdenum and reconstitute molybdoenzyme activity? Many papers have claimed that the utilization of tungsten in place of molybdenum produces inactive proteins, for examples the membrane-bound DMSO reductase from *E. coli* (Bue *et al.*, 1999), periplasmic nitrate reductases from *E. coli* and *Paracoccus pantotrophus* (Gates *et al*., 2003), formate dehydrogenase from *Methanobacterium formicium* (May *et al*., 1988). Hagen (2011) claimed that tungstate can be used instead of molybdenum, especially in anaerobic archaea and some bacteria.

1.10 *E. coli* nitrate reductase

The respiratory nitrate reductase, NarGHJI, is a membrane bound nitrate reductase, which its active site is present in the *E. coli* cytoplasm and is also known as nitrate reductase A, NRA (Forget, 1974; Enoch and Lester, 1975; Chaudhry and Macgregor, 1983; Blasco, *et al*., 1990; Iobbi-Nivol *et al.*, 1990; Cole, 1996).

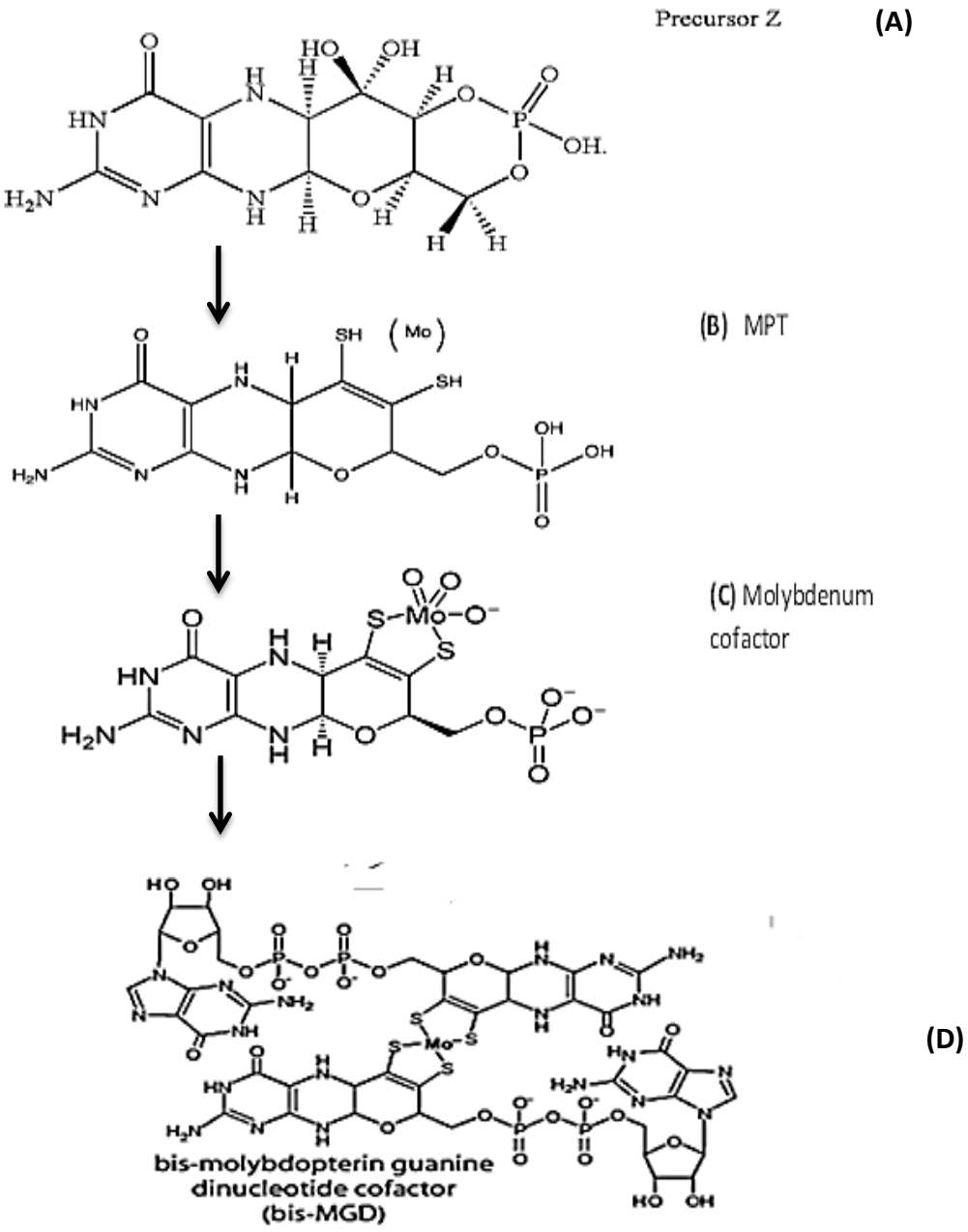


Figure 1.7: Schematic representation of the Structure of molybdopterin cofactor

The molybdopterin precursor Z (A), the molybdopterin (MPT) (B), the molybdenum cofactor (C) and the molybdenum guanine dinucleotide form (bis-MGD) (D) (Hille, 1996; Rajagopalan and Johnson, 1992).

This protein consists of three specific protein subunits: α, β and γ (Figure 1.8), which are encoded by *narG*, *narH*, and *narI*, respectively (Berk *et al.*, 1995b), in addition to the complementary soluble protein subunit, NarJ (Eaves *et al*., 1998). The *narI* gene encodes the transmembrane protein subunit that is located in the bacterial cytoplasmic membrane, and holds the remainder of the nitrate reductase to the cell membrane. Reduced NADH is able to donate electrons through the quinol pool to the γ – protein via a high potential *b*-type haem group that is coordinated in the γ-subunit (Hackett and Bragg, 1993*).*

Continuous reduction of the haem group in the γ–protein and oxidation of the quinol pool causes the flow of two protons (H^+) into the periplasm, and thereby the generation of a proton motive force or Δp (Garland *et al.*, 1975). The flow back of protons across inner membrane leads to production of ATP from ADP catalysed by a specific enzyme called ATPase (Garland *et al*., 1975). Before the electrons eventually reach the catalytic α-subunit, NarG, via a β-subunit that contains two types of [Fe-S] clusters including a [3Fe-4S] centre and a [4Fe-4S], they are transferred to a lower potential haem group of the γ – protein (Figure 1.8). The α-protein NarG containing Mo-*bis*-MGD accepts two electrons to catalyse the reduction of the nitrate to nitrite (Squire, 2009). Nitrate reductase Z (NRZ) is the second nitrate reductase that is an analogue to NarGHI (Iobbi *et al*., 1987). This enzyme has been found in few microorganisms such as *E. coli* and *Salmonella typhi* (Bonnefoy *et al*., 1997). There is very considerable similarity between this enzyme and NarGHI, particularly regarding to the α, β and γ subunits (Blasco *et al*., 1990; Bonnefoy *et al*., 1997). The α-subunit of NarZYW contains the molybdopterin cofactor that corresponds to the α-subunit of NarG (Blasco *et al*., 1990).

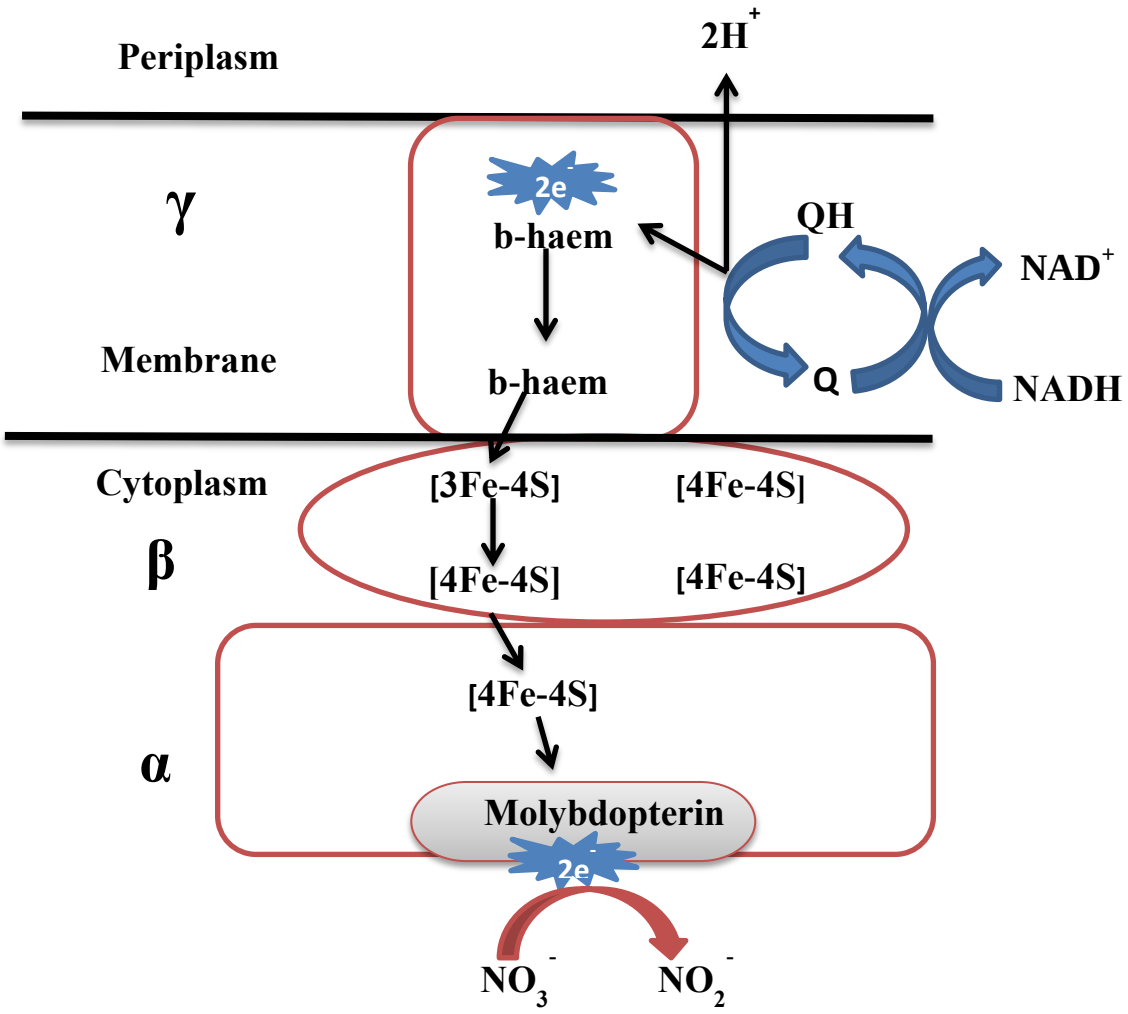


Figure 1.8: A model of electrons transport and reduction of nitrate to nitrite in *E. coli* by the nitrate reductase, NarGHI

Reduced NADH donates two electrons to the γ-subunit of the nitrate reductase A via the quinol pool. The molybdenum cofactor α-subunit of NarG accepts the electrons that are transferred via the two haem groups in the γ-subunit to the [Fe-S] of the β-subunit. In the cytoplasm nitrate is reduced to nitrite, which is coupled to the expulsion of $2H^+$ into the periplasm and the production of Δp.

Work by Iobbi *et al*. (1990) and Chang *et al*. (1999) shows that NarZYW is unlike NarGHI in the regulation of gene expression. It is known that the induction of NarGHI increases in the presence of nitrate during anaerobic conditions, while NarZYW expression is undetectable whether nitrate or oxygen are present or absent, and it is induced at a very low level during exponential phase (Iobbi *et al*., 1987). However, expression studies revealed that expression of this protein is highly increased at the beginning of the stationary growth (Chang *et al.*, 1999). Moreover, it has been reported that NarZ plays a significant role in conferring a selective advantage for bacterial survival during stationary phase, or growth in media lacking the main nutrient factors (Iobbi *et al*., 1987; Clegg, 2002).

In *E. coli*, the periplasmic enzyme, Nap, is encoded by the *nap* operon, which consists of seven genes, *napFDAGHBC.* These genes are expressed under nitrate limiting condition (Potter *et al*., 1999; Wang *et al*., 1999; Stewart *et al*., 2002). There are four common Nap proteins, NapA, NapB, NapC and NapD and in all bacteria, they have an essential role in Nap activity (Figure 1.9). NapA is a periplasmic protein, and has been reported to be bound to a critical Mo-bis-MGD cofactor and iron-sulfur clusters [4Fe-4S]. NapB is a di-haem protein that donates electrons to NapA for reduction of nitrate to nitrite. NapC is a tetra-haem cytochrome that is located in the cytoplasmic membrane and operates as a quinol dehydrogenase (Breks *et al*., 1995a; Brondijk *et al.,* 2004). NapD is situated in the cytoplasm, where it might be involved in maturation of NapA (Potter and Cole, 1999; Brondijk *et al.*, 2004). A consequence of the reduction of nitrate during anaerobic conditions is the generation of toxic nitrite that builds up rapidly in nitrate–respiring bacteria (Cole, 1996). In order to counter this potent product, *E. coli* readily pumps nitrite transported by NarK or NirC proteins of the bacterial cell.

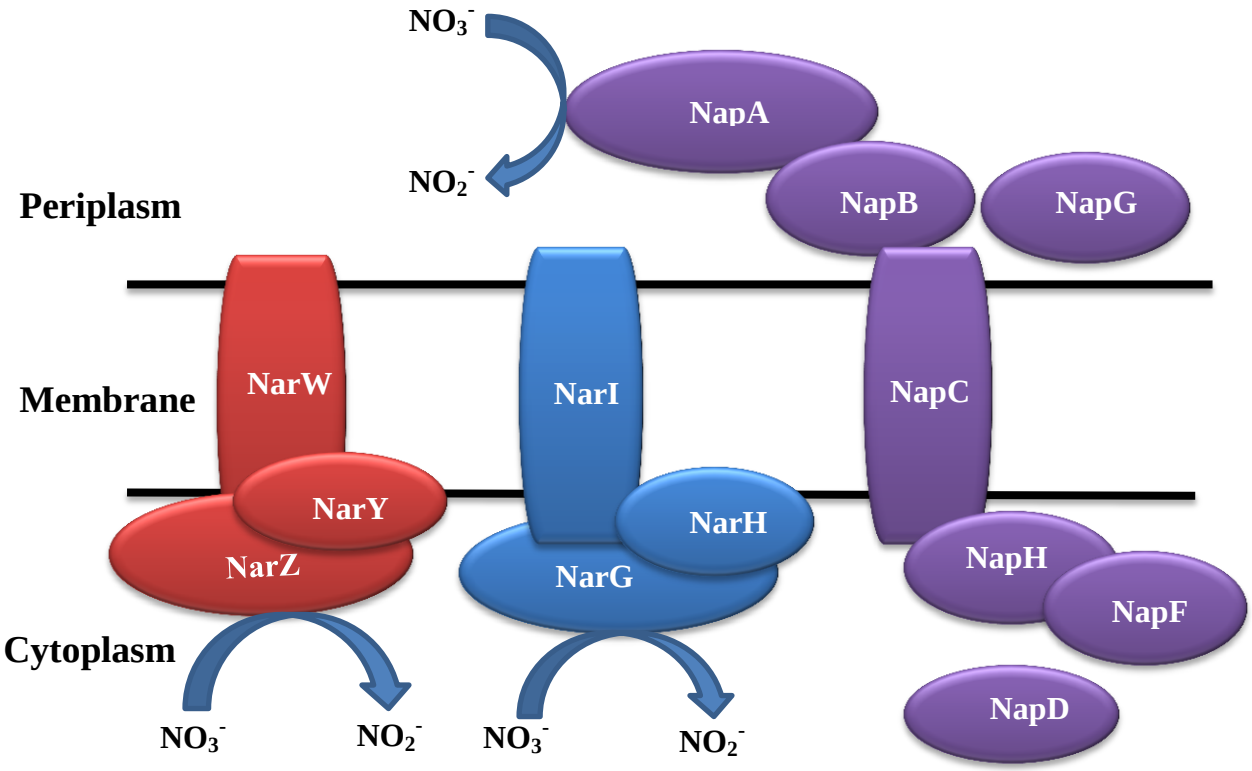


Figure 1.9: A scheme showing the cellular location of nitrate reductases subunits in *E. coli*

Membrane associated NarGHI is shaded blue, membrane bound nitrate reductase NarZYW is shaded red and periplasmic nitrate reductase is shaded purple. The chaperone components of these complexes were not shown for simplicity of the diagram.

Alternatively, any nitrite present in the cytoplasm can be reduced whether it is produced endogenously as a side- product of nitrate reduction, or it is present freely in the environment (Clegg *et al*., 2002; Jia *et al*., 2009).

1.11 *E. coli* nitrite reductase

In addition to nitrate reduction, *E. coli* is able to reduce nitrite to ammonia by two independent enzymes, NrfA and NirB (Page *et al*., 1990; Wang and Gunsalus, 2000). NirB is a soluble cytoplasmic enzyme that contains sirohaem as cofactor, which can catalyse the 6 electron reduction of nitrite to ammonia, but does not generate a membrane potential (Δp) during this process. This protein accepts electrons from reduced NADH in order to reduce nitrite. In *E. coli*, nitrite is accumulated when nitrate is reduced. But this accumulated nitrite can be ejected by NarK (Cole, 1996). The regulation of *nirB* operon coordinates with synthesis of nitrate reductase respiratory system (NarGHJI) in order to detoxify all accumulated nitrite during nitrate reduction. (Browning *et al*., 2005).

Expression of the *E. coli nirB* operon is increased in oxygen-limited environments and in the presence of nitrate and nitrite, and it totally depends on FNR, as well as NarL and NarP, which are responsible for activation of this operon as previously discussed. The cytochrome-*c* nitrite reductase, NrfA, located in the periplasm is encoded by the *nrf* operon. This enzyme utilizes formate as electron donor during nitrite reduction, which leads to generation of a proton gradient (Pope and Cole, 1982). The Nrf protein complex consists of five subunits including the *c* type cytochrome (NrfA), which also is called cytochrome $c_{552.}$ It forms a complex with a periplasmic pentahaem cytochrome (NrfB) (Hussain *et al*., 1994). The NrfC subunit is a membrane -bound non-haem iron-sulfur protein containing four [4Fe-4S] centres. NrfD is characterised as a membrane-bound quinol dehydrogenase (Berks *et al.,* 1995a;

Poock *et al*., 2002). The oxidation of formate by formate dehydrogenase and the coordinate of the reduction of nitrite by NrfA via quinol pool cause the generation of a membrane potential (Δp) (Hussain *et al*., 1994; Eaves *et al*., 1998).

1.12 *E. coli* encounters oxidative and nitrosative stress

To survive, *E. coli* has the ability to defend itself against reactive oxygen species (ROS) or reactive nitrogen species (RNS) that damage proteins, lipids and DNA (Tucker *et al*., 2010; Corker and Poole, 2003). Host defence mechanisms such as neutrophils or phagocytic cells are able to produce superoxide radicals (O_2^-) (Miller and Britigan, 1997). These radicals then might be converted by superoxide dismutase to other ROS namely hydrogen peroxide (H_2O_2) (Fang, 2004).

E. coli encounters NO and RNS from different sources and its formation occurs under aerobic or anaerobic conditions during infection. Aerobically, mammalian immunity cells such as macrophage, release NO as part of their defence mechanism when infected (Baek *et al*., 1993; Arkenberg *et al.*, 2011). It has been shown that the concentration of NO produced from activated macrophages is up to 10 µM (Raines *et al*., 2006).

In contrast, under anaerobic conditions most of the nitric oxide is generated as side-products of *E. coli* metabolism via a 1 e^- reduction from nitrite. It has been claimed that the mechanism of endogenous NO production might be attributed to the action of both nitrite reductases, NirB and NrfA (Corker and Poole, 2003; Weiss, 2006). However, many studies reported that the membrane-associated nitrate reductase, NarG, is responsible for the generation of most of the NO (Metheringham and Cole, 1997, Taverna and Sedgwick, 1996, Ralt *et al.*, 1988, Calmels *et al.*, 1988, Ji and Hollocher, 1988; Gilberthorpe and Poole, 2008; Vine *et*

al., 2011b). This toxic product is also an intermediate during denitrification. Finally, other types of bacteria that live in the same environment of *E. coli* are able to generate NO as well, (Crawford and Goldberg, 1998; Fang, 2004; Corker and Poole, 2003).

1.13 Damage caused by nitric oxide

Nitric oxide is a free radical gas that causes enormous cellular damage. This reactive molecule is able to bind to other chemicals in the cell in order to produce secondary toxic products. For example, NO reacts with metal centres of some citric acid cycle enzymes such as aconitase A and fumarase B by inactivating their labile iron-sulfur centres and form dinitrosyl-iron complex (DNIC) in the place of their iron-sulfur cluster. In this case, the damage is called nitrosylation (Kennedy *et al*., 1997; Gardner *et al.,* 2001; Cruz-Ramos *et al*., 2002; Justino *et al* 2009). In the presence of oxygen, NO can form various reactive intermediates (RNS) including peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), nitrosyl cation (NO^+), nitroxyl anion (NO^-) and nitrogen dioxide (NO_2) that have unique chemistries that participate in spectrum damage of the cell (Hughes, 1999).

Peroxynitrite is a highly reactive and toxic product produced from reacting NO with the superoxide radical. The pathogenicity of *E. coli* and other related species might be attributed to the ability of *E. coli* to resist the toxicity of peroxynitrite. N_2O_3 is a powerful nitrosating agent generated from reacting NO with oxygen. This toxic product causes deleterious effects to DNA and thereby leads to mutation (Spek *et al*., 2001). Nitrosyl cation can be generated from NO in the presence of O_2 and metal ions. This occurs because NO is unable to react directly with thiols when both O_2 and metal ions are unavailable. The biological nitrosation process is a reaction of the NO^+ group with a nucleophile, such as a sulfur atom from cysteine

or thiol, to form S-NO which are referred to as S-nitrosothiols (RSNO) such as S-nitrosocysteine (SNO-Cys) (Arnelle and Stamler, 1995; Williams, 1999; Pullan *et al.*, 2007).

1.14 Catalytic enzyme aconitase and its sensitivity to O_2 and NO

Aconitase or aconitate dehydratase has a vital role in the tricarboxylic acid (TCA) cycle. It catalyses the reversible isomerization of citrate to isocitrate via aconitate (Krebs and Hlzach, 1952). *E. coli* contains two distinct aconitases, AcnA and AcnB (Brock *et al*., 2002). Aconitase A is highly induced during oxidative stress *in vivo* (Cunningham *et al*., 1997), but, Aconitase B is more sensitive to oxidative stress *in vivo* (Varghese *et al.*, 2003; Brock *et al*., 2002). AcnB enzyme plays a major role in the TCA cycle (Gruer and Gruest, 1994; Gruer *et al*., 1997). Expression of this enzyme is highly induced during the exponential phase of growth and decreases in the stationary phase. In conditions with low iron concentrations, AcnB becomes inactive rapidly (Varghese *et al.*, 2003).

In *E. coli* under oxidative stress, O_2^- mediates inactivation of the aconitase iron-sulfur cluster through oxidation of the $[4Fe\text{-}4S]^{2+}$ clusters and forms an unstable $[4Fe\text{-}4S]^{3+}$ cluster, which breaks down rapidly to $[3Fe\text{-}4S]^{1+}$. The activity of aconitase in a cell-free extract of a strain defective in superoxide dismutase and grown aerobically was lower than in the parent strain (Gardner and Fridovich, 1991). Regarding nitrosative stress, aconitase B is also sensitive to inactivation even by low concentrations of NO (Ren *et al*., 2008; Justino *et al*., 2007). Work by Gardner *et al*. (1997) suggested that aconitase inactivation is due to the S-nitrosation of its iron-sulfur cluster. On the other hand, addition of Fe^{2+} and cysteine causes reactivation of purified aconitase *in vitro* (Morrison, 1954). Several studies proposed that aconitase damaged by both O_2^- and NO can be reactivated *in vitro* by decreasing the

surrounding level of O_2^-. However, the level of reactivation achieved by decreasing the amount of NO was less compared with O_2^- (Gardner *et al.*, 1997).

1.15 Catalytic enzyme fumarase and its sensitivity to O_2 and NO

Fumarase, also known as the fumarase hydratase, plays an important role in the TCA cycle. Fumarase catalyses the reversible hydration of fumarate to malate (Woods *et al*., 1988; Kim *et al*., 2007). In *E. coli,* three types of fumarase are known, FumA, FumB, and FumC. FumA and FumB are class one fumarases encoded by the genes *fumA* and *fumB,* respectively. FumA is the major TCA cycle enzyme that is highly expressed during aerobic growth, while its expression is repressed by anaerobosis and glucose (Woods and Guest, 1987). However, induction of FumB and FumC are strongly increased under anaerobic conditions (Woods and Guest, 1987; Tseng *et al*., 2001).

Class I fumarase has been found in enterobacteria such as *Salmonella* and *Klebsiella.* Class II fumarases does not contain iron, therefore they are insensitive to oxidative stress (Flint, 1994). FumB and FumA contain an O_2-sensitive catalytic [4Fe-4S] cluster, which is converted to inactive [3Fe-4S] cluster by oxidative agents such as superoxide (O_2^-) (Flint *et al*., 1992; Flint and Allen, 1996).

FumC serves as a support enzyme in the cell when both FumA and FumB are destroyed by oxidative stress (Tseng *et.al,* 2001; Lara *et al*., 2006). Additionally, the effects of reactive nitrogen species such as NO also have been investigated, with results indicating that NO reacts with the iron-sulfur cluster of FumB, thereby yielding inactive enzyme (Justino *et al*., 2007).

1.16 NO detoxification systems in *E. coli*

To overcome the toxicity of NO generated either by immune cells or by its own metabolism, *E. coli* has evolved a set of mechanisms to deal with the deleterious effects of NO. It is known to have three NO detoxification enzymes, namely, flavohaemoglobin, HmpA, periplasmic cytochrome-*c* nitrite reductase, NrfA, and flavorubredoxin NorV (Saraiva *et al.*, 2004; Vicente *et al.*, 2008; Vine and Cole, 2011). Poole *et al*. (1996) noticed that transcription of the *hmp* gene is highly increased with addition of NO, suggesting that Hmp is implicated in protection against NO. This implication was confirmed when the *hmp* mutant strain showed great sensitivity to NO (Stevanin *et al.*, 2002).

Under aerobic conditions, Hmp is able to oxidize nitric oxide to nitrate. Conversely, under anaerobic conditions, Hmp reduces nitric oxide to a less toxic product, nitrous oxide, but it is apparent that the role of this enzyme is only minor in terms of protecting the bacterium from toxic NO generated under these conditions (Hausladen *et al.*, 1998; Kim *et al.*, 1999; Gardner and Gardner, 2002). Hmp belongs to a family of flavohaemoglobins and its N-terminal haem binding domain linked with a flavin-binding domain plays a critical role in NO reduction (Hernandez-Urzua *et al*., 2003). The expression of the *hmp* gene is repressed by the transcription factor FNR that binds over the -10 sequence of the *hmp* promoter, thereby preventing RNAP binding (Poole *et al*., 1996).

The *hmpA* promoter is a target for another transcription regulator, namely the repressor protein, NsrR, which represses its expression. However, its expression is relived after exposure to NO (Bodenmiller and Spiro, 2006; Pullan *et al*., 2007). Previously in this chapter, it has been described that NrfA contributes to the reduction of nitrite to ammonia, and also generates cytotoxic NO as a side-product (Poock *et al.*, 2002; Van Wonderen *et al*., 2008). It

has been shown that external NO formed by the host or nearby microorganisms would be decomposed by NrfA as an initial defence mechanism (Van Wonderen *et al*., 2008). Poock *et al.* (2002) showed that a strain with *nrfA* deleted has high sensitivity to NO. Moreover, any NO that evades reduction by NrfA and passes into the cytoplasm will be trapped by the third NO reductase (NorV), which belongs to the A-type flavoprotein family. In *E. coli*, this protein consists of NorV, encoded by the *norV* gene, and its associated flavo-protein oxidoreductase NorW, encoded by the *norW* gene, and together they are able to reduce NO to N_2O (Tucker *et al*., 2005). The nitric oxide sensitive transcription regulator, NorR, regulates expression of the *norVW* operon (Gardner and Gardner, 2002; Justino *et al*., 2005). NorR is a σ^{54}-dependent transcription factor that recognises DNA binding sites in the *norVW* promoter located at position -24 and -12 (Barrios *et al*., 1999; Shingler, 2012).

1.17 The *E. coli* nitric oxide repressor, NsrR

The nitric oxide-sensitive repressor protein, NsrR, encoded by the *nsrR* gene, was first identified in *Nitrosomonas europaea* (Beaumont *et al.,* 2004). It belongs to the Rrf2 family of transcription regulators that contain iron-sulfur clusters. In *E. coli*, NsrR is directly responsive to NO as it contains an NO-sensitive [2Fe-2S] cluster, which is required for DNA binding. In the presence of NO under anaerobic conditions, the iron-sulfur centre is nitrosylated, which in turn relieves repression and then allows transcription of genes implicated in the nitrosative stress response (Tucker *et al*., 2008; Tucker *et al*., 2010). Some studies have demonstrated that the regulatory protein, NsrR, represses several genes in *E. coli* MG1655, which include *hmpA; ytfE; hcp-hcr; nrfA; ygbA* and *yeaR-yoaG* (Bodenmiller and Spiro, 2006, Filenko *et al.,* 2007). It has been shown that this NO-sensitive repressor binds to an inverted repeat sequence ($GATGN_{11}$ CATC), which overlaps the -10 element of promoters (Bodenmiller and

Spiro, 2006). The NsrR protein has been found in a wide variety of bacteria, including the human pathogen *Neisseria gonorrhoeae,* in which NsrR also contains an NO-sensitive [2Fe-2S] centre (Isabella *et al.,* 2009). Likewise, the NsrR proteins of Gram positive bacteria such as *Bacillus subtilis* and *Streptomyces coelicolor*, which contain [4Fe-4S] and [2Fe-2S] clusters respectively, have also been shown to be sensitive to NO (Tucker *et al*., 2008; Yukli *et al*., 2008). The NsrR of *Streptomyces coelicolor* has been purified and exposed to NO, which in turn caused nitrosylation of its cluster and resulted in the loss of DNA binding affinity *in vitro* (Tucker *et al*., 2008).

1.18 The *E. coli* oxidative stress regulator, OxyR

The transcription factor OxyR regulates the expression of many genes involved in oxidative stress. It is a member of the LysR family of bacterial transcription factors, and is composed of 305 amino acid residues (Christman *et al*., 1989; Tao *et al*., 1989). It has two domains, an N-terminal domain that carries a helix-turn-helix motif and a C-terminal domain, which are joined by a flexible linker (Kullik *et al*., 1995; Zaim and Kierzek, 2003). OxyR is activated by H_2O_2, and then causes formation of a disulphide bond between Cys-199 and Cys-208 (Zheng *et al.,* 1998). Thus, the oxidized form of OxyR is able to induce RNA polymerase to bind to the target gene and activate transcription (Tao *et al*., 1993; Kullik *et al*., 1995). The binding of OxyR to the DNA occurs as a dimer (Muraoka *et al*., 2003; Gusarov and Nudler, 2012). It has been shown that OxyR is able to interact with other members of the LysR family in order to form heterodimers. However, the physiological importance of this process is still unknown (Knapp and Hu, 2010). The innate immune system activates macrophages to produce RNS and ROS (Nathan, 1995) and this imposes oxidative and nitrosative stress in order to kill the invading pathogens (Nunoshiba *et al*., 1993; MacMicking *et al*., 1995). During exponential growth of *E. coli,* the induction of *oxyR* is highly controlled by the

cAMP-activated CRP protein. In *E. coli* and *Salmonella typhimurium*, OxyR regulates expression of several genes including hydroperoxidase/catalase (*katG*), alkyl hydroperoxide reductase (*ahpCF*), and glutathione reductase (*gorA),* and hence expression of these genes provides protection against H_2O_2 (Christman *et al* 1985; Storz and Imlay, 1999).

Moreover, S-nitrosylation of *E. coli* OxyR under nitrosative stress causes up-regulation the expression of genes to protect against nitrosative stress (Hausladen 1996; Seth *et al*,. 2012), as the amount of S-nitrosylated proteins in *oxyR* mutant was more than in the wild type strain during anoxic conditions in the presence of nitrate (Seth *et al.*, 2012). Thus, OxyR has been illustrated to function as a sensor of both oxidative and nitrosative stress. Therefore, to confirm whether OxyR has any role in the nitrosative stress, the function of the OxyR in protection against nitrosative stress will be investigated during this study.

1.19 The *E. coli* di-iron protein, YtfE

A microarray paper first showed that the *E. coli ytfE* gene was highly expressed upon exposure to nitrosative stress (Justino *et al.*, 2005). The product of the *E. coli ytfE* gene is a di-iron protein, YtfE, that was renamed RIC for the repair of iron-centres of proteins damaged by oxidative or nitrosative stress (Justino *et al.*, 2006; 2007; Overton *et al.*, 2008). The regulator of nitrosative stress, NsrR, but not NarXLQP, regulates transcription of *ytfE* (Bodenmiller and Spiro, 2006). YtfE is a cytoplasmic protein that can be found in all enterobacteria (Justino *et al*., 2006). It has been reported that the *ytfE* mutant LMS4209 was more sensitive to reactive nitrogen species and is impaired in the activity of some proteins containing iron-sulfur clusters (Justino *et al.*, 2006). Furthermore, when grown anaerobically the *ytfE* mutant showed highest damage of the iron-sulfur clusters of both aconitase B and fumarase A enzymes in the presence of NO and H_2O_2. The growth defect was complemented

using plasmid encoded YtfE. The activities of both enzymes were restored *in vitro* by incubation with purified YtfE. Therefore, it was suggested that YtfE protein has a crucial role in repairing of iron-sulphur centres damaged by nitrosative stress (Justino *et al*., 2007). This study also has showed that YtfE provides protection against oxidative stress. YtfE homologues are widely distributed proteins in nature. They can be found in higher eukaryotes, parasites and bacteria. Analysis of the amino acid sequences of YtfE proteins from different organisms showed that carboxylate (aspartate and glutamate) and histidine residues could form ligands for the dinuclear non-heam iron centre (Justino *et al*., 2006; Overton *et al*., 2008). Electron paramagnetic resonance spectroscopy (EPR) confirmed that *E. coli* YtfE contains di-iron sites (Justino *et al*., 2006). Studies of the *E. coli* YtfE by resonance Raman and extended X-ray absorption fine structure (EXAFS) showed that YtfE contains a non-haem dinuclear iron centre with μ-oxo and μ-carboxylate bridging ligands and six histidine residues coordinating the iron ions (Figure 1.10; Todorovic *et al*., 2008).

The orthologue of YtfE in *Staphylococcus aureus* is been called ScdA while in *Neisseria gonorrhoeae* it is called DnrN (Overton *et al*. (2008). Synthesis of both of these proteins was induced in the presence of both NO and H_2O_2 to confer protection against oxidative and nitrosative stress. In *Neisseria gonorrhoeae*, there was more damage to transcription factors, FNR and NsrR, by stress in the *dnrN* mutant compared to that in the parental strain. The activity of iron-sulfur proteins such as fumarase was far lower in the staphylococcal *scdA* mutant compared to that in the parental strain (Overton *et al*., 2008). Several studies have suggested that the possible role of YtfE is to repair iron centres by donating Fe to the damaged [Fe-S] clusters (Justino *et al*., 2009). A recent study showed that YtfE slowly releases iron atoms that can be transferred to iron-deficient Fe-S clusters.

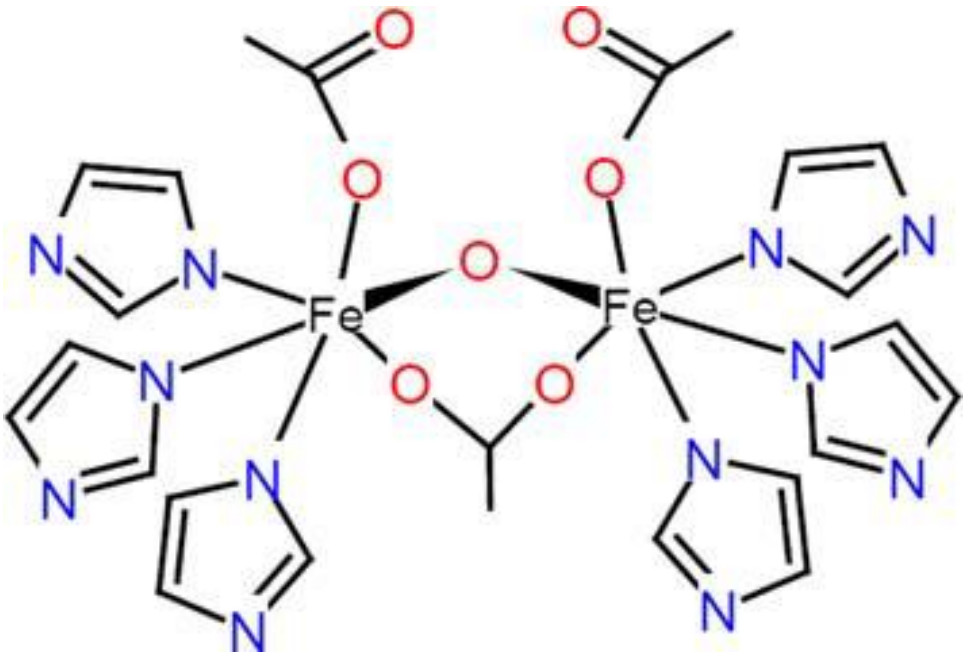


Figure 1.10: A model of the YtfE di-iron centre in *E. coli* in its oxidized state

The two upper oxygen ligands of both iron atoms can also be provided by only one carboxylate group, thus leading to the formation of a second μ-carboxylate bridge. The figure and ligand were taken from Todorovic e*t al*. (2008).

However, the exact mechanism of this repair has not yet been resolved (Nobre *et al*., 2014). Vine *et al.* (2010) completed experiments with a strain that carried a *ytfE* mutation LMS4209 and found an additional deletion of another 126 genes in addition to the *ytfE* mutation. The secondary deletion also included genes that are essential for molybdopterin synthesis. For instance, the deletion included the *moa* and *mod* operons required for molybdopterin synthesis, and hence for formate dehydrogenase, nitrate reductase and TMAO reductase synthesis. The discovery of these deleted genes easily explained that growth defective of the *ytfE* strain, LMS4209 reported by Justino *et al*. (2006). The deletion also included the *hcp-hcr* operon (Vine *et al.*, 2010). In view of these more recent observations, the aim of this study was to define more precisely the biochemical function of YtfE in protecting bacteria against nitrosative stress.

1.20 The *E. coli* hybrid cluster protein, HCP

The hybrid cluster protein, Hcp, encoded by the *hcp* gene, contains two types of clusters. Ones is a [4Fe-4S] cubane cluster and the other is the hybrid [4Fe-2O-2S] iron-sulfur oxygen cluster (Figure 1.11) that is so far unique in biology (Arendsen *et al*., 1998; van den Berg *et al*., 2000). Although the crystal structure of the *Desulfovibrio vulgaris* Hcp was determined in 1998, its biochemical function is unknown (Arendsen *et al*., 1998; Aragão *et al*., 2008). In *E. coli*, the *hcp* gene is closely linked to a gene for an NADH-dependent Hcp reductase, Hcr (van den Berg *et al*., 2000). NADH donates electrons to Hcr that transfers them to the hybrid cluster of Hcp via the [2Fe-2S] of the Hcr (van den Berg *et al*., 2000). Expression of this two-gene operon is highly activated only during anaerobic growth in the presence of nitrate and nitrite (van den Berg *et al*., 2000). Pullan *et al*. (2007) confirmed experimentally that expression of the *hcp* is upregulated under anaerobic conditions in the

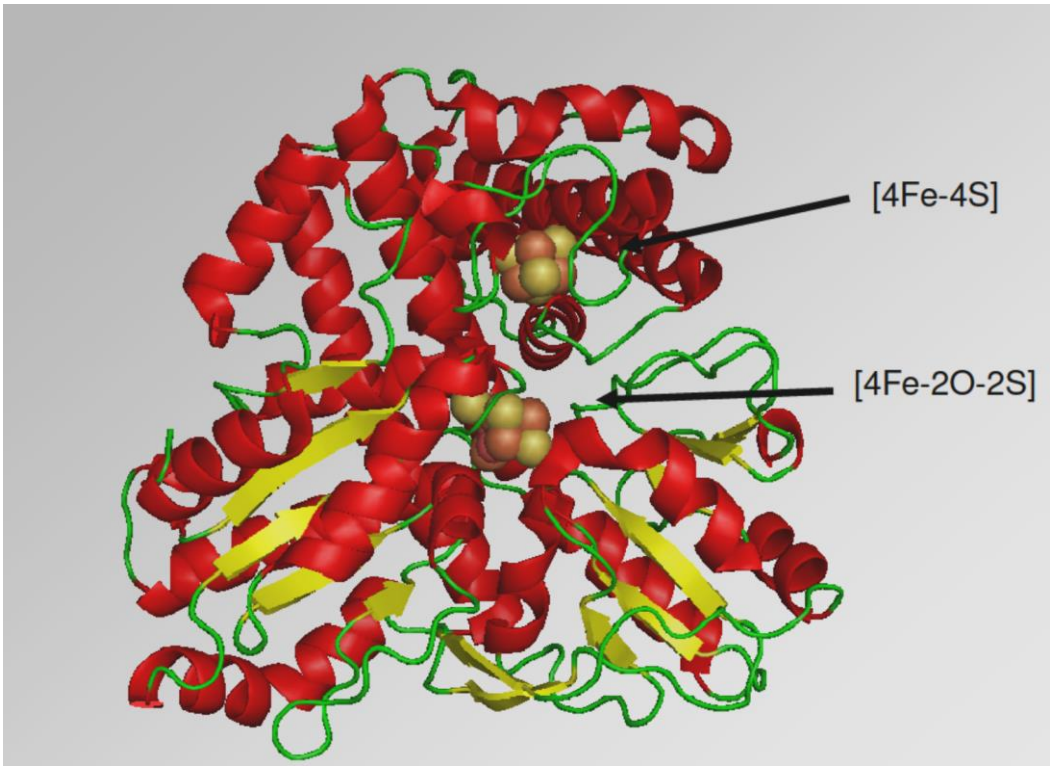


Figure 1.11: The structure of the hybrid cluster protein, HCP.

The two iron-sulfur clusters in Hcp protein purified from *Desuphovibrio vulgaris*. The arrows indicate the position of both clusters, [4Fe-4S] cubane cluster and [4Fe-2O-4S] iron–sulfur- oxygen clusters. The crystal structure was downloaded from RSCB protein database and modelled using PyMOL modelling software. Squire (Ph.D Thesis, 2009).

presence of NO. Filenko *et al*. (2005) used an *hcp::lacZ* fusion reporter plasmid to show that FNR and nitrate/nitrite regulators NarL and NarP regulate Hcp synthesis in *E. coli*. It has been found that FNR regulates *hcp* transcription, which binds to a site centered around -72.5 upstream of the transcript start site (Chismon *et al*, 2010).

In another study by Chismon (2011) demonstrated that when FNR is active, *hcp* transcription can be induced during anaerobic growth even in the absence of nitrate and nitrite. However, the addition of nitrate and nitrite led to the maximal activity. The *hcp* gene is repressed by the nitric oxide-sensitive repressor, NsrR (van den Berg *et al*., 2000; Constantinidou *et al*., 2006; Seth *et al*., 2012). A study by Filenko *et al.* (2007) confirmed that *hcp* transcription is repressed 15-fold by NsrR. The repressor function of NsrR is inactivated by nitric oxide (Tucker *et al*., 2008; Yuki *et al*., 2008), so highest levels of *hcp-hcr* expression are induced by nitrosative stress during anaerobic growth (Filenko *et al*., 2007; Vine *et al*., 2010). Almeida *et al*. (2006) showed that *hcp* can be induced by OxyR in the presence of H_2O_2. In contrast, Seth *et al*. (2012) showed that H_2O_2 failed to induce *hcp* under both aerobic and anaerobic conditions. The same study by Seth *et al.* (2012) showed that OxyR regulates the expression of *hcp*, which in turn provides protection against S-nitrosylation in *E. coli* under oxic and anoxic conditions in the presence of nitrate.

Several studies suggested that Hcp is a hydroxylamine reductase (Wolfe *et al*., 2002; Cabello *et al*., 2004). In contrast, Almedia *et al*. (2006) proposed that expression of Hcp is induced in response to oxidative stress; thereby it can function as a peroxidase. The addition of hydroxylamine to the growth medium did not induce the expression of the *hcp* (Filenko *et al*., 2007; Squire, PhD Thesis, 2009). It has been suggested that genes encoding Hcp are widely distributed in anaerobic or facultatively anaerobic bacteria, but absent from aerobic

bacteria (Vine and Cole, 2012). *E. coli* mutated in *hcp-hcr* and all nitrite and NO reductases showed poor growth in the presence of nitrite (Vine and Cole, 2011; Vine, PhD Thesis, 2012). Furthermore, it was shown that Hcp plays a key role in survival of *Desulfovibrio vulgaris* in macrophages (Figueiredo *et al.,* 2013). Vine (2012) claimed that Hcp might remove the toxic product that is generated by YtfE during nitrosative stress.

Work by Wang *et al*. (2016, in submission) showed that *Escherichia coli* Hcp is a high affinity nitric oxide (NO) reductase that, in the absence of NorVW, is essential for survival under physiologically relevant conditions of nitrosative stress. Deletion of *hcp* results in extreme sensitivity to NO during anaerobic growth and inactivation of the iron-sulfur proteins, aconitase and fumarase. For these reasons, the role of Hcp along with YtfE in dealing with RNS will be investigated during this study.

1.21 Aims of this study

The previous study by Seth *et al*. (2012) found that OxyR increases transcription from the *hcp* promoter under both aerobic and anaerobic conditions during conditions of nitrosative stress. An initial aim of this study is to confirm this hypothesis by constructing a new strain that is defective in the *oxyR* gene. The effect of OxyR at the *hcp* promoter in this strain will be compared with the parental strain RK4353.

NsrR is a specific sensor of NO that has been shown to negatively regulate the expression of several genes implicated in the nitrosative stress response: *ytfE*; *hmp*; *ygbA*; *nrfA*; *hcp-hcr*; and *yeaR-yoaG* (Bodenmiller and Spiro, 2006, Filenko *et al.,* 2007). A further aim of this work is to investigate whether the NsrR protein autoregulates the *nsrR* promoter by constructing *nsrR*::*lacZ* fusion plasmid. Several studies claimed that FNR could also sense

NO. To confirm these findings, experiments will be designed to reveal whether FNR is a physiologically relevant NO sensor.

The product of the *E. coli ytfE* gene is a di-iron protein, YtfE (or RIC for the repair of iron-centres of proteins damaged by oxidative or nitrosative stress). YtfE has been extensively implicated in the repair of iron-sulfur centres of proteins damaged by nitrosation (Justino *et al.*, 2006; 2007; 2009; 2014; Overton *et al*., 2008). Although it is clear that YtfE slowly releases iron atoms that can be transferred to iron-deficient Fe-S centres, the exact mechanism of this repair has not yet been resolved (Nobre *et al.*, 2014). In view of these more recent observations, the final aim of the work described in this study will be to define more precisely the biochemical function of YtfE in protecting bacteria against nitrosative stress.

CHAPTER TWO-MATERIALS AND METHODS

2.1 Bacterial media

2.1.1 Liquid media

E. coli was routinely grown in Luria-Bertani (LB) broth (Oxoid) that was prepared by dissolving 20 g l^{-1} tryptone, 10 g l^{-1} yeast extract and 10 g l^{-1} NaCl in distilled water. Minimal salts medium (MS) was prepared by adding 4.5 g l^{-1} KH_2PO_4, 10.5 g l^{-1} K_2HPO_4, 1 g l^{-1} NH_4SO_4, 0.05 g l^{-1} $MgCl_2$, 1 ml ammonium molybdate, 1 ml sodium selenite and *E. coli* free salts (1ml.l^{-1}) sequentially to distilled water. The *E. coli* free salts contains 82 g l^{-1} $MgCl_2.7H_2O$, 10 g $MnCl_2.4H_2O$, 4 g l^{-1} $FeCl_2.6H_2O$, 1 g l^{-1} $CaCl_2.6H_2O$ and concentrated HCl per litre of distilled water. This medium then was supplemented with 20 mM TMAO, 20 mM fumarate, 0.4% (v/v) glycerol as carbon source and 5% LB. For the transformation procedure, superoptimal broth (SOC) medium was made by dissolving 20 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 0.85 g l^{-1} NaCl, 0.185 g ml^{-1} KCl, 2.03 $MgCl_2.6H_2O$, 2.46 g 1^{-1} $MgSO_4.7H_2O$, and 3.6 g 1^{-1} glucose in distilled water.

2.1.2 Solid media

Nutrient agar plates were made by dissolving 28 g·l^{-1} nutrient agar (Oxoid, Hampshire, UK) in distilled water. Lennox agar (LA) was prepared freshly each day to get overlay plates that were used for transduction. This medium was prepared by dissolving the appropriate amount of LB agar in distilled water and supplemented with 1.25% bacteriological agar. MacConkey agar (MCA) contained 50 g l^{-1} of MacConkey lactose agar dissolved in distilled water.

2.2 Sterilising media

All media (liquid and solid) were dissolved completely and the pH was adjusted to 7.0 then media were autoclaved at 121ºC for 15 minutes. Before the addition of antibiotics, all solid media were autoclaved and cooled to 80ºC, and then around 20 ml of cooled molten media was added to a 30 ml petri dish. All plates were allowed to solidify at room temperature, and then were stored at 4ºC for 4 weeks.

2.3 Antibiotics

Tetracycline (Tet)

Stock solution of 10 mg ml^{-1} was prepared by dissolving the powdered antibiotic in 50 % v/v ethanol followed by filter sterilisation. This antibiotic was sterilised by filtration using 0.2 µm sized filter membrane and stored at -20ºC for 4 weeks. The stock solution was used at a concentration of 35 µg ml^{-1}.

Kanamycin (Kan)

Stock solution of 100 mg ml^{-1} was made by adding an appropriate amount of antibiotic to distilled water and sterilised by filtration. It was stored at 4ºC and used at a concentration of 25 µg ml^{-1}.

Chloramphenicol (Chlor)

Stock solution of 50 mg ml^{-1} was prepared by dissolving antibiotic powder in ethanol and was sterilised by filtration. Chloramphenicol was stored at -20ºC and used at a concentration of 30 µg ml^{-1}.

Ampicillin (Amp)

Stock solution of 100 mg ml^{-1} was made by dissolving it in distilled water and it was filter sterilised. This antibiotic was stored at 4ºC and used at a concentration of 100 mg ml^{-1}.

2.4 Buffers and solutions used in this study

2.4.1 General buffers

Phosphate buffer (50 mM)

It was made by dissolving 7.26 g l^{-1} K_2HPO_4, and 1.13 g l^{-1} KH_2PO_4 in distilled water, its pH was adjusted to 7.4.

X-Gal (50 mg ml^{-1})

5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) was prepared by dissolving 100 mg in 2 ml of N,N, dimethyl-formamide and stored at -20º C.

IPTG (0.1 M)

Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was made by dissolving 1.2 g in 50 ml of distilled water. It was sterilised by filtration and stored at 4ºC.

2.4.2 Buffers and solutions for β-galactosidase assay

Z buffer

This buffer was prepared by dissolving 0.75 g l^{-1} KCl, 0.25 g l^{-1} $MgSO_4.7H_2O$, 8.55 g l^{-1} Na_2HPO_4, 4.87 g l^{-1} $NaH_2PO_4.2H_2O$ in distilled water and adjusted to pH 7.0. β-mercaptoethanol (2.7 ml l^{-1}) was added before use.

1xA, Potassium phosphate buffer

1xA buffer was made to a 0.1 M concentration by dissolving 11.6 g l^{-1} K_2HPO_4 and 4.54 g l^{-1} KH_2PO_4 in distilled water and adjusted to pH 7.0.

Sodium deoxycholate (1%)

Sodium deoxycholate (1 g) was dissolved in 100 ml of distilled water.

Orthonitrophenyl-β-D-galactosidase (ONPG)

To make a solution with a final concentration of 13 mM of ONPG, 39.2 mg was dissolved in 10 ml of 0.1 M of 1×A buffer and the pH adjusted to 7.0. A solution of sodium carbonate (Na_2CO_3) (1 M) was used to stop the reaction.

2.4.3 Buffers for making competent *E. coli*

TFB 1

TFB 1 buffer contained 12.1 g l^{-1} RbCl (100 mM), 9.9 g l^{-1} $MnCl_2.4H_2O$ (50 mM), 2.9 g l^{-1} potassium acetate (30 mM), 1.1 g l^{-1} $CaCl_2$ (10 mM) and 150 ml glycerol (15%) in distilled water and was calibrated to pH 5.8 water. It was sterilised by filtration and stored at 4ºC.

TFB 2

TFB 2 buffer contained MOPS 2.1 g l^{-1} (10 mM),1 2 g l^{-1} RbCl (10 mM), 8.3 g l^{-1} $CaCl_2$ (75 mM) and 150 ml glycerol (15%) in distilled and was calibrated to pH 6.8 water. It was sterilised by filtration and stored at 4ºC.

2.4.4 Buffers for agarose gel electrophoresis of DNA

5xTBE buffer

A 5x Stock was made by using 0.44 M Tris-HCl, 0.445 M boric acid and 0.01 M EDTA, and then adjusted to pH 8.0.

1xTBE buffer

To make 1xTBE for agarose gel electrophoresis, stock solution 5xTBE was diluted to a 1: 5 solution.

2.5 Buffers and stock solutions for aconitase, fumarase and folin-phenol assays

Buffers for these assays are listed in Table 2.1.

2.6 Strains

The bacterial strains used in this work are listed in Tables 2.2 and 2.3.

2.7 Bacterial preparation for long term storage

In order to make a stock of the strain needed to be stored, a single colony of the strain was inoculated into 1 ml LB and was then aerated vigorously at 37ºC for 4-8 hours until the optical density had reached 0.7-0.8. The culture (100 µl) was added to 0.3 ml of sterilised glycerol (50%v/v) in a sterile screw-top 2 ml tube, and was stored at -80ºC.

2.8 Plasmids

QIAprep Spin Miniprep Kit was used to isolate all plasmids used in this study as described by the manufacturer. Plasmids used in this work are listed in Table 2.4.

2.9 Competent cells and transformation

2.9.1 Competent *E. coli* preparation

A single colony was used to inoculate 5 ml of LB medium in 25 ml conical flask containing antibiotic as necessary. This culture was aerated overnight at 37ºC. A preheated 100 ml of sterilised LB medium in 250 ml conical flask containing the relevant antibiotic was inoculated with 1 ml of overnight culture. The culture was further shaken at 37ºC for approximately 90-120 minutes, until the optical density had become between 0.4-0.7.

The culture was cooled for 5 minutes on ice and then transferred to sterile centrifuge tubes. The cells were collected by centrifugation at low speed (4000 g, 5 minutes, 4ºC). The supernatant was discarded and the cells were resuspended in cold TFB 1 buffer which was prepared as described previously. The suspension was kept on ice for 90 minutes, and the cells were then pelleted once again by centrifugation at 4000 g, 5 minutes, 4ºC. The supernatant was discarded carefully and the cells were resuspended in TFB 2 buffer. The competent cells were stored in 100-200 µl aliquots in sterile microcentrfuge tubes and were snap-frozen in liquid nitrogen. The competent cells were then stored at -80ºC.

Table 2.1**: Stock and solution for fumarase, aconitase and folin-phenol assays**

Name	Ingredients
Buffers and stock solutions for fumarase assay	
Phosphate buffer (50 mM)	1.13 g l^{-1} KH_2PO_4 and 7.26 g l^{-1} K_2HPO_4 (pH 7.4)
Nicotinamide adenine dinucleotide (NAD^+)	40 mg ml^{-1}
Sodium fumarate (1M)	160 g l^{-1}
Glutamate oxaloacetate aminotransferase	0.3 mg.l^{-1}
Sodium glutamate (1M)	18.71 g l^{-1}
Buffers and Stock solutions for aconitase assay	
phosphate buffer (50 Mm)	1.13 gl^{-1} KH_2PO_4 and 7.26 g l^{-1} K_2HPO_4
aconitase assay buffer (50 mM Tris-Hcl, 0.6 $Mncl_2$)	6.057 g of Tris were dissolving in 1 L of distilled water, the pH was adjusted to 7.4 and 120 g was then added.
1 M sodium citrate	0.5 g l^{-1}
0.1 M $NADP^+$	76.5 mg ml^{-1}
Buffers and Stock solutions for folin-phenol assay	
2% sodium carbonate	2 g per 100 ml in sodium hydroxide (1 M)
Sodium hydroxide (1 M)	39.99 g l^{-1}
1% copper sulphate	1 g in 1 ml distilled water
2% sodium tartrate	2 g $m1^{-1}$ distilled water
Folin phenol reagent	10 ml in distilled water
Bovine serum albomine	0.01 g ml^{-1} distilled water

Table 2.2: *E. coli* K-12 strains used in this study

Strain	Description	Source
RK4353	Δ*lacU169araD139 rpsL gyrA*	(Stewart and McGregor,1982)
JCB4022	RK4353 Δ*narG::ery* Δ*napAB*	(Potter *et al* ., 1999)
JCB5222	RK4353 Δ*nsrR::kan*	RK4353 transduced with P1 propagated on JCB 3901
JCB5211	RK4353 Δ*ytfE::cat*	RK4353 transduced with propagated on LMS 4209
JCB5000	RK4353 Δ*hcp::cat*	(Vine, PhD Thesis, 2012)
JCB5228	RK4353 Δ*ytfE* Δ*hcp::cat*	(Vine, PhD Thesis, 2012)
JCB5249	RK4353 Δ*nirBDC* Δ*nrfAB* Δ*norV* Δ*hmp*::*kan*^{S}	(Vine, PhD Thesis, 2012
JCB5210	RK4353 Δ*nirBDC* Δ*nrfAB* Δ*norVW* Δ*hmp*::*kan*	(Vine, PhD Thesis, 2012)
JCB5250	RK4353 Δ*nirBDC* Δ*nrfAB* Δ*norV* Δ*hmp* Δ*hcp*::*cat*	(Vine, PhD Thesis, 2012)
JCB5253	RK4353 Δ*nirBDC* Δ*nrfAB* Δ*norV* Δ*hmp* Δ*hcp*::*cat*^{S}	(Vine, PhD Thesis, 2012)
JCB5251	RK4353 Δ*nirBDC* Δ*nrfAB* Δ*norV* Δ*hmp* Δ*fnr*::*cat*	(Vine, PhD Thesis, 2012)
JCB5257	RK4353 Δ*nirBDC* Δ*nrfAB* Δ*norV* Δ*hmp* Δ*ytfE*::*cat*	(Vine, PhD Thesis, 2012)
JCB5260	RK4353 Δ*nirBDC* Δ*nrfAB* Δ*norV* Δ*hmp* Δ*hcp* Δ*ytfE::cat*	(Vine, PhD Thesis, 2012)
JCB5270	RK435 3Δ*narZ* Δ*narG* Δ*nirCBD* Δ*nrfAB* Δ*norV* Δ*hmp* Δ*hcp* Δ*ytfE*::*cat*	(Vine, PhD Thesis, 2012)
JCB5280	RK4353 Δ*narZ* Δ*narG* Δ*nirBCD* Δ*nrfAB* Δ*norV* Δ*hmp* Δ*hcp*::*cat*	(Vine, PhD Thesis, 2012)
JCB5265	RK4353 Δ*narZ* Δ*narG* Δ*nrfAB* Δ*norV* Δ*hmp* Δ *nirBCD::kan*^{s}	(Vine, PhD Thesis, 2012)
JM109	*recA* Δ*lacZ*	Promega

Table 2.3: Strains with multiple mutations made by bacteriophage P1 transduction for this work

Strain	Description	Source
JCB5301	RK4353 Δ*oxyR::Kan*	*E. coli* MG1655 Δ*oxyR::kan* transduced with P1 propagated on RK4353
JCB5300	RK4353 Δ*narG*::*ery* Δ*napAB* Δ*ytfA::cat*	Δ*ytfE::cat* transduced with P1 propagated on JCB4022
JCB5272	RK4353 Δ*narZ* Δ*narGHJI* Δ*nrfAB::cat*^{S} Δ*hmp::kan*^{S} Δ*norV::cat*^{S}Δ *nirBDC::kan*^{S} Δ*ytfE::cat*	Δ*ytfE::cat* transduced with P1 propagated on JCB5265
JCB5259	RK4353Δ*narZ* Δ*narGHJI* Δ*nrfAB::cat* Δ*hmp::kan*^{S} Δ*norV::cat*^{S}	Derivative from cured JCB5256
JCB5266	RK4353 Δ*narZ* Δ*narGHJI* Δ*nrfAB::cat* Δ*hmp::kan*^{S} Δ*norV::cat*^{S} Δ*ytfE::cat*	RK4353 Δ*ytfE::cat* transduced with P1 propagated on cured JCB5259
JCB5218	RK4353 Δ*nirBDC*::*kan*S Δ*nrfAB*::*cat*^{S} Δ*norV*::*cat*^{S} Δ*hmp*::*kan*^{S} Δ*nsrR::kan*	JCB5222 transduced with P1 propagated on JCB5249
JCB5258	RK4353 Δ*nirBDC::kan*^{s} Δ*nrfAB::cat*^{s} Δ*norV::cat*^{s} Δ*hmp::cat*^{s} Δ*nsrR::kan*	JCB5222 transduced with P1 propagated on JCB5253
JCB52701	RK4353 Δ*narZ* Δ*narGHJI* Δ*nrfAB::cat* ^{s}Δ*hmp::kan*^{s} Δ*norV::cat*^{s} Δ*nirBDC::kan* ^{s}Δ*hcp::cat* Δ*mobA::kan*	JCB5270 transduced with P1 propagated on BW25113 *mobA*
JCB5111	RK4353 Δ*nirBDC* Δ*nrfAB* Δ*norV* Δ*hmp*::*kan*^{S} Δ*fnr::cat*	JCB3911 transduced with P1 propagated on JCB5249
JCB5112	RK4353 Δ*nirCBD* Δ*nrfAB* Δ*norV* Δ*hmp* Δ*hcp*::*cat*^{S} Δ*fnr::cat*	JCB3911 transduced with P1 propagated on JCB5253

Table 2.4: Plasmids and primers used in this study

Plasmids	Descriptions	Reference
pNF383	The *hcp* regulatory region was cloned into pRW50 to produce an *hcp::lacZ* fusion. Tet^R.	(Filenko *et al*., 2007)
pSP01	*phmp* was cloned into the EcoR1/ HindIII restriction sites of pRW50. Tet^R.	(Vine. PhD Thesis, 2012)
pRW50	*LacZ* fusion vector was utilized for cloning promoters as EcoR1/HindIII fragments. It was used for all β-galactosidase Assay. This plasmid contains the RK2 origin of replication and encodes Tet^R (Figure 2.1).	(Lodge *et al*., 1992)
pGEM-Teasy	The vector was used to clone PCR products and for blue/white screening of recombinants (Figure 2.2).	(Promega)
pPL341	pBR322 carrying *hmp* under its own promoter. Encodes Amp^R.	(Vasudevan *et al.*, 1991)
pLG339	Low copy number plasmid encodes *nrfA* under its own promoter. Encodes $Tet.^R$	(Stoker *et al*., 1982)
pBKB1	*pnsrR* cloned into EcoR1/HindIII restriction sites of pRW50. Ter^R.	(This work)
pCP20	Expression of the Flp recombinase gene (exo) was under the control of heat sensitive promoter. Encodes Amp^R Cm^R.	(Dastenko and Wanner, 2000)
pAA182-PnorV	Encodes *norV* under its own promoter. Amp^R	(Justino *et al.*, 2005)
pACYC184	This vector encodes *ytfE* under its own promoter. It is low copy number and contains the p15A1 origin of replication. Encodes Tet^R and Cm^R (Figure 2.3).	(Rose, 1988)

pET-24a	High copy number plasmid encodes a T7 promoter and the expression of the target gene is under the control of T7 polymerase. It is also encodes *lacI* sequence and encodes Tet^R (Figure 2.4).	Novagen

Primer name	
ytfE forward	5' ACC GAC AGT GAT TCT CC 3'
ytfE reverse	5' GTA TCG CAA ACA GCG TC 3'
nsrR forward	5' CTG CGC GAC CCG TTC GAC GCG 3'
nsrR reverse	5' CGC CCT TCT GGC AAT GAC GCC 3'
oxyR forward	5'ATT TGA GCC TGG CTT ATC GC 3'
oxyR reverse	5'ACA AAT CGT CGG CAT GAA CG 3'
mobA forward	5' ATG TCA TCC GTC AAA TCC GC 3'
mobA forward	5' TGG TGT TTC CGT CAT CAA GG 3'

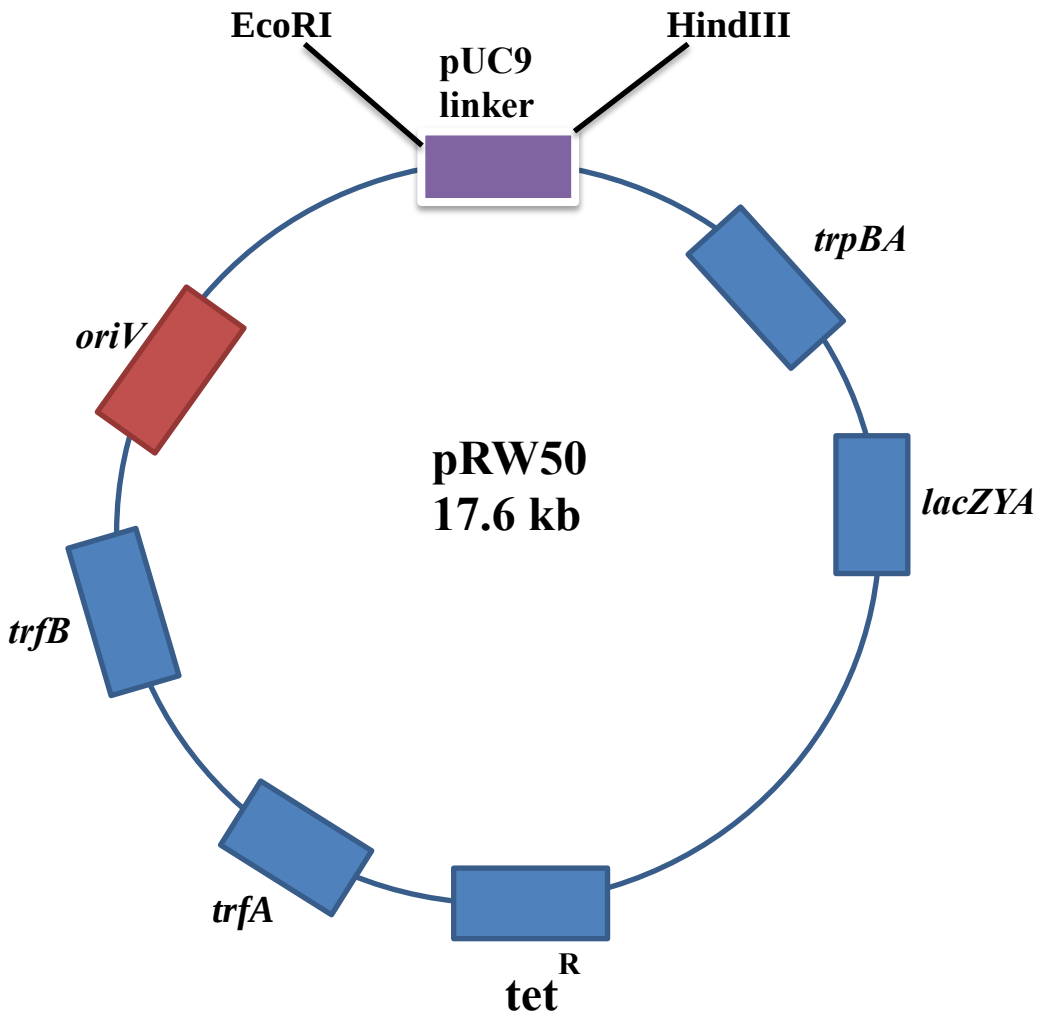


Figure 2.1: A schematic of the *lac-Z* fusion vector, pRW50

A schematic representation of the plasmid pRW50, EcoRI-*Hin*dIII promoter fragments are cloned into the pUC9 linker (shaded purple) as transcriptional fusions to the *trapBA* and *lacZYA* operon (coloured blue), such that expression of the *lac* genes are dependent on the cloned promoter. Also shown are the tetracycline resistance gene (*tet*R), the origin of replication (*oriV)*, the plasmid replication genes *trfA* and *trfB*, and *trpBA*, which forms an operon with *lacZYA*.

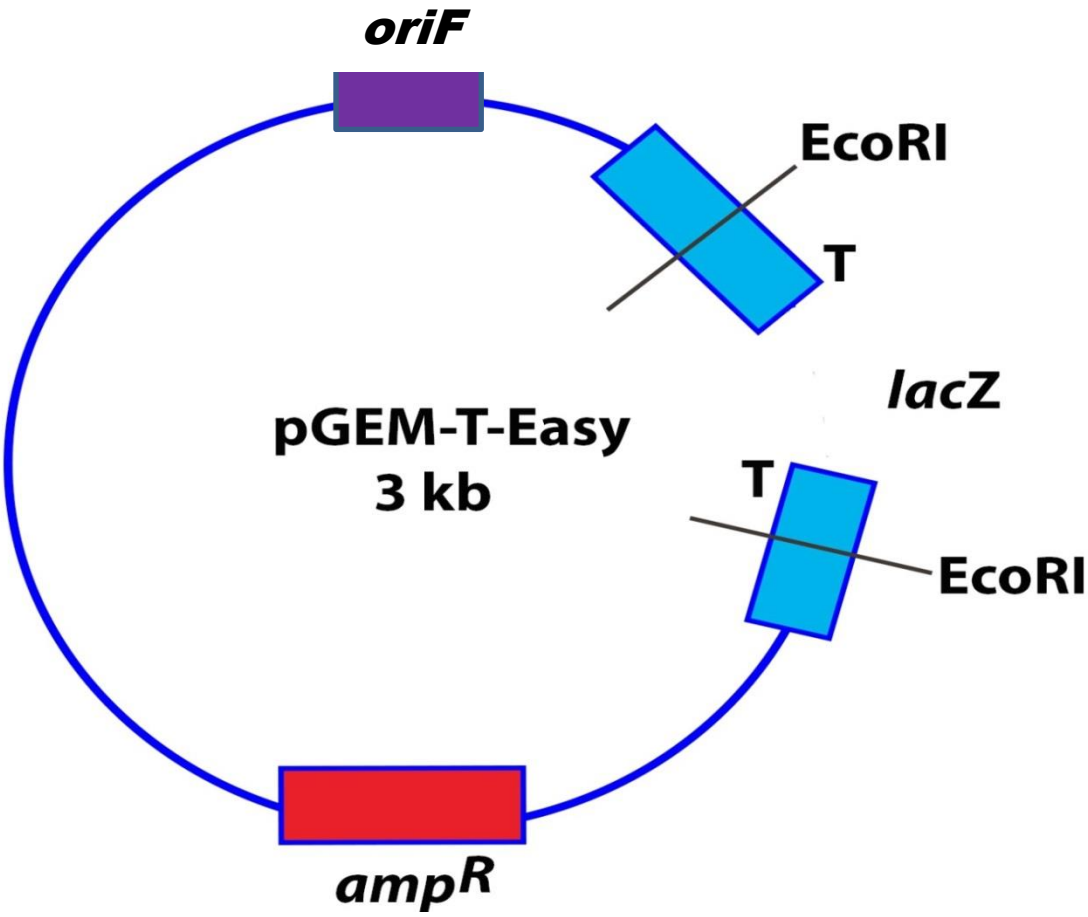


Figure 2.2: Diagram of high copy number cloning vector, T-easy

The plasmid pGEM-T Easy was used for cloning of PCR products for amplification, and blue/white screening of recombinants. The plasmid is approximately 3 kb in size and carries an ampicillin resistance marker. This plasmid is supplied in linear form with the β-galactosidase gene interrupted by a poly-linker with an oligo-dT tail on either end. This enable the direct cloning of PCR products with oligo-dA tails directly into the vector. The insertion of a PCR fragment causes an interruption of the plasmid-encoded *lacZ* gene allowing for the selection of transformants with correct insert through blue/white screening.

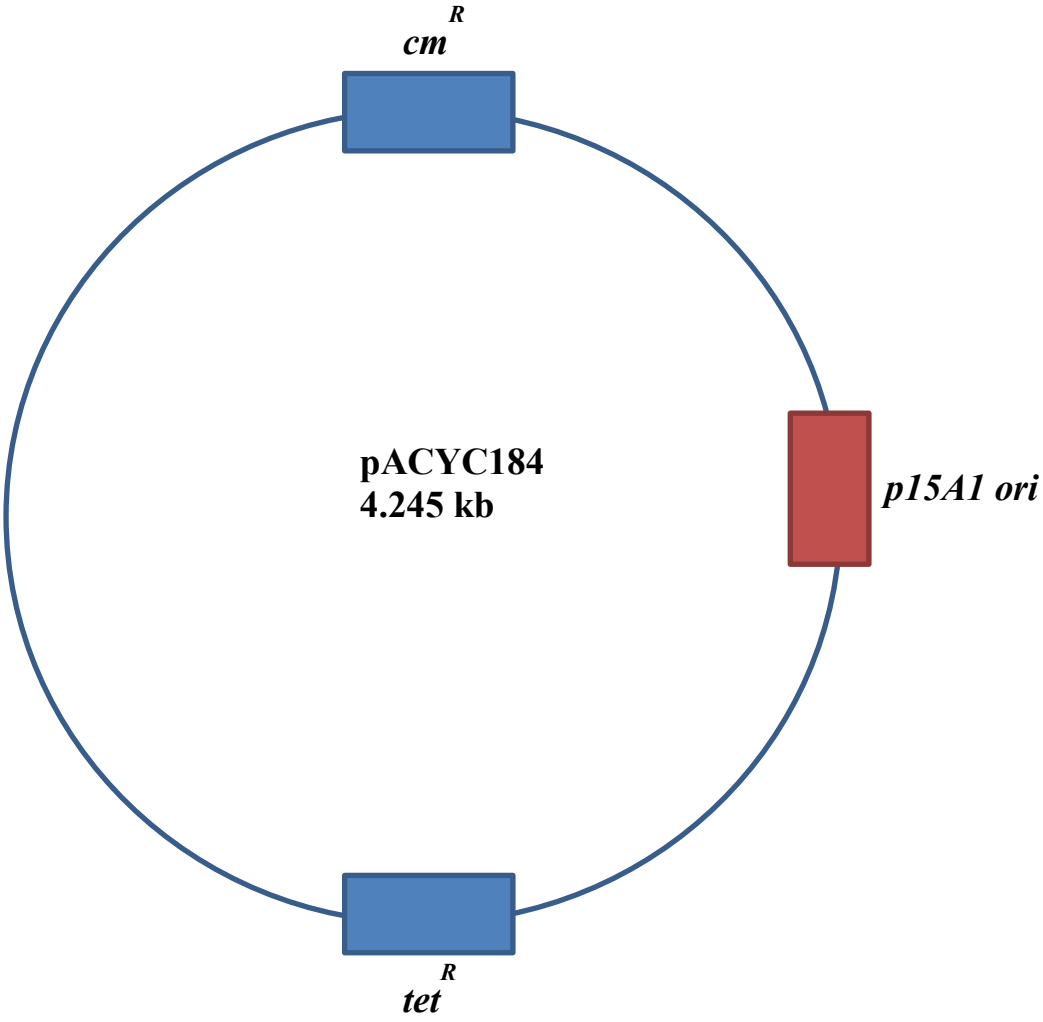


Figure 2.3: The low-copy cloning vector, pACYC184

This figure shows a map of pACYC184, which encodes p15A1 origin of replication in *E. coli*. It is a low copy number vector, at approximately 15 copies per cell. Also shown are the tetracycline and chloramphenicol resistance (*tet*^{R}, *cm*^{R}).

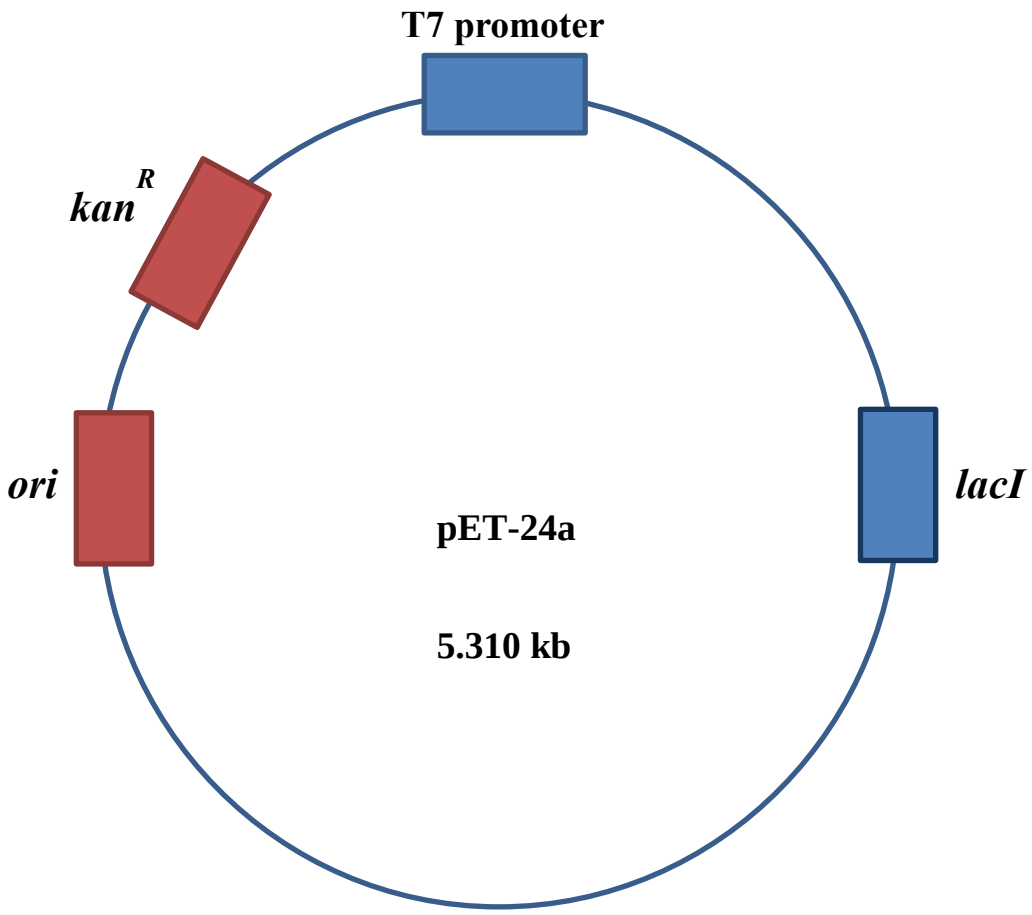


Figure 2.4:.The high-copy cloning vector, pET-24a

A schematic representation of the plasmid pET-24a, encoding N-terminal T7 Tag and C-terminal His Tag sequence. This plasmid carry kanamycin resistance marker (*kan*^{R}). Also shown are the *lacI* coding sequences and *ori* (3227) for replication.

2.9.2 Transformation of competent *E. coli* with plasmid DNA

Competent cells (50 to 100) µl were transferred to 1.5 ml microcentrfuge tube containing 1 to 5 µl of plasmid DNA, the mixture was mixed thoroughly and kept on ice for 40 minutes. After incubation on ice, the cells were heat shocked at 42ºC in water bath or heating block for 3 minutes, and then incubated on ice for 2 minutes. The LB (1 ml) or SOC (1 ml) medium was added immediately to the culture, followed by shaking for 1 to 2 hours at 37ºC. The transformant cells (50 to 100 µl) were plated out on agar plates containing the appropriate selective antibiotic.

2.10 Knockout of the antibiotic resistance cassette using pCP20

In order to remove the desired antibiotic resistant cassette, the 50 µl of Kanamycin or chloramphenicol resistant competent cells that contain FRT-flanked marker with PCP20 from which the antibiotic resistant cassette was to be removed were mixed with 1 µl pCP20 plasmid DNA. This mixture was incubated for 40 minutes on ice and then was heat shocked for 2 minutes at 42ºC, then re-incubated on ice for another 2 minutes. The SOC or LB (1 ml) medium was added to the culture in sterile test tube followed by incubation at 30ºC with aeration for 1 to 2 hours. The transformation mixture (50 to 100 µl) was plated out on nutrient agar plates supplemented with carbenicillin and incubated at 30ºC overnight. Suitable candidates were purified on NA and reincubated at 42ºC overnight in order to inhibit replication of pCP20. To test the removal of antibiotic resistant cassette and plasmid encoded carbenicillin resistance, single colonies were picked and replica plated onto three plates, nutrient agar without any antibiotic, nutrient agar with carbenicillin, nutrient agar with kanamycin or chloramphenicol. Colonies that were sensitive to both antibiotics but that grew

on NA were tested by colony PCR, using primers that flank the gene of interest, for the presence of an 82 bp scar in place of the kanamycin or chloramphenicol cassette.

2.11 *E. coli* chromosomal mutations transfer by P1 transduction

Donor strains containing the selectable mutation of interest to be moved were grown aerobically at 37ºC in 1 ml of LA supplemented with 2 mM $CaCl_2$ until the optical density had reached 07.0.8. Lennox agar, was prepared, autoclaved and then cooled to 58ºC. This medium was supplemented with 0.2% (w/v) glucose and 2 mM $CaCl_2$ then poured into plate and allowed to set at room temperature. The remaining 1 ml of this agar which was still warm was added to 6 sterile test tubes containing 2 ml of LB supplemented with 2 mM $CaCl_2$ at 45ºC. Stock bacteriophage P1 was serially diluted from 10^{-1} to 10^{-6} in LB-Ca^{2+}. The bacterial donor (0.1 ml) and diluted bacteriophage were pipetted into the agar aliquot in each tube. All the tubes were then vortexed immediately and poured onto the Lennox agar plates in order to create a soft agar overlay. The plates were then incubated at 37ºC overnight without inversion. On the same day, 1 ml LB-Ca^{2+} was inoculated with the recipient bacteria.

The following day, the lacy plates with plaque formation that were very close together were selected and bacteriophage P1 was harvested by adding 2 ml LB-Ca^{2+} to the plates. A dry glass spreader was used to break the soft agar layer which was homogenized in 1 ml of ice cold chloroform in a cold hand homogeniser. The bacterial slurry was centrifuged at 3000 rpm for 15 minutes and the top layer containing the P1 was mixed with 1ml of chloroform and stored at 4ºC for transduction. An overnight culture of the recipient strain was added to 20 ml LB-Ca^{2+} and grown at 37ºC until the optical density had reached 0.7-0.8. After incubation, the recipient culture was divided into three sterile test tubes and collected by centrifugation at 3600 rmp for 3 minutes. The pellets were then resuspended in LB-Ca^{2+} (0.5 ml) and then

diluted 0.1 ml P1 was added to the first tube, 0.1 ml undiluted P1 was added to the second tube and nothing to the control tube. The three tubes were incubated at 37ºC for 18 minutes in order to allow for efficient transduction and then 1 ml of sterile MS was used to re-suspend the cells. The cells were collected by centrifugation at 36000 rmp for 3 minutes, the supernatant was discarded and the pellet was resuspended in 4 ml of sterile MS. Cells were pelted once again by centrifugation at 36000 rpm for 3 minutes and the method was repeated 3 times to remove excess P1. At the end of the last wash, the pellets were resuspended in just 2 ml of sterile LB with no $CaCl_2$ and incubated at 37ºC for 1 to 2 hours. Finally, 0.1 ml of the bacterial suspension was plated out onto a dry agar plate containing the appropriate antibiotic.

2.12 Construction of *ytfE* mutation in RK4353 Δ*narZ* Δ*narGHJI* Δ*nrfAB::cat*^{s} Δ*hmp::kan*^{s} Δ*norV::cat*^{s} Δ*nirBDC::kan*^{s}

P1 transduction was used to transduce the *ytfE*::*cat* mutation from strain RK4353 Δ*ytfE*::*cat* to RK4353 Δ*narZ* Δ*narGHJI* Δ*nrfAB::cat*^{s} Δ*hmp::kan*^{s} Δ*norV::cat*^{s} Δ*nirB::kan*^{s}. ,JCB5265. The deletion of the presence of the chloramphenicol resistance cassette (*cat)* in the place of the *ytfE* gene was detected by testing purified chloramphenicol resistant colonies using PCR with two primers, one that binds upstream and the other that binds downstream of *ytfE*. Agarose gel electrophoresis was used to visualize the amplified PCR products. The transductants contained the correct chloramphenicol cassette in place of *ytfE* to produce strain RK4353 Δ*narZ* Δ*narGHJI* Δ*nrfAB::cat*^{s} Δ*hmp::kan*^{s} Δ*norV::cat*^{s} Δ*nirBDC::kan*^{s} Δ*ytfE::cat*, JCB5272, which was used for further investigation.

2.13 Construction of *ytfE* mutation in RK4353 Δ*narZ*Δ *narGHJI* Δ*nrfAB::cat*^{s} Δ*hmp::kan*^{s} Δ*norV::cat*^{s}

In order to construct this strain, bacteriophage P1 transduction was used to transduce the *ytfE*::*cat* mutation from RK4353 Δ*ytfE*::*cat* to cured RK4353*ΔnarZΔnarGHJI ΔnrfAB::cat*^{s} *Δhmp::kan*^{s} *ΔnorV::cat*^{s} *hcp*$^{+}$*nirB*$^{+}$, JCB5259. After the required strain was constructed, colony PCR was used to check that the *ytfE* mutation was correct. As this transductant was correct, the new strain RK4353 Δ*narZ ΔnarGHJI* Δ*nrfAB::cat*^{s} *Δhmp::kan*^{s}Δ*norV::cat* *hcp*$^{+}$*nirB*$^{+}$*ΔytfE::cat* strain, JCB5266 was available for further experiments. The details of each transduction are listed in Table 2.3.

2.14 Nitric oxide preparation

To prepare a vessel of NO at pH 3 anaerobically, 2 ml of distilled water at PH 3 was transferred to a small bijoux bottle and sealed with turnover rubber stopper. This was covered with silicone grease and sealed with parafilm to avoid oxygen leaking into the bijoux bottle, which was connected to the apparatus placed in the fume cupboard. Two needles were inserted into the rubber stopper, the short one was inserted into the space above the liquid while the long one was inserted into the liquid part of the vessel. The needles were attached to two cylinders of nitrogen and nitric oxide (BOC, gases, Surrey, UK) by silicone tubes through 3 conical flasks of distilled water, 1 M $KMnO_4$ and NaOH. The nitrogen cylinder was switched on and left to bubble into the bijoux bottle for 30 minutes, then the clamp of nitrogen was switched off, and the clamp of nitric oxide was opened for 30 minutes. The nitric oxide saturated water (NOSW) with 2 mM was produced.

2.15 Growth assays in the presence of nitrosative and oxidative stress

In order to determine the effect of nitrosative stress on growth, all strains used in this study were grown in 100 ml MS medium in a 100 ml conical flask supplemented with 5% LB, 0.4% glycerol, 20% mM sodium fumarate and 20 mM TMAO. All cultures then were inoculated with overnight culture, which was grown in 5 ml LB in 25 conical flask at 37ºC overnight with aeration. Growth of most cultures was started with high cell density 0.2. The anaerobic cultures in presence or absence of relevant antibiotics were incubated without shaking at 37ºC for one hour for it to adapt properly to anaerobic conditions before addition of nitrate, nitrite and NO which was added sequentially every 30 minutes, .or until the optical density at 650 nm reached 0.2 in case of other cultures. However, the cultures that used for oxidative stress were supplemented with hydrogen peroxide when the optical density had reached to 0.3. Aerobic cultures were incubated at 37ºC with aeration at 100 rpm. The effect of both nitrosative and oxidative stress was monitored on supplemented growth compared to unsupplemented control.

2.16 β-galactosidase activity assay

Jayaraman *et al*. (1987) method was used for β-galactosidase assay. Cells were grown in medium as mentioned previously with 35 $\mu g.ml^{-1}$ tetracycline. Aerobic cultures were aerated at 100 rpm at 37ºC while anaerobic cultures were incubated statically at 37ºC and both cultures were monitored until the optical density at 650 nm had reached 0.15 or 0.2 respectively. After the culture had reached an appropriate optical density, the culture (1 ml) was transferred to a clean test tube incubated in ice. Cells were lysed with 30 µl of toluene and 30 µl of sodium deoxycholate (1%) and then aerated at 37ºC for 20 minutes to allow toluene to evaporate and then the lysate was incubated on ice. The lysate was diluted 1-5 fold

in potassium phosphate buffer (1 x A) and added to 1.6-1.9 ml Z buffer. The total volume of 2 ml was prewarmed at 37ºC. ONPG (13 mM, 0.5 ml) was added and mixed by vortexing. The reaction was incubated at 37ºC until the yellow colour was noted. After the development of yellow colour, the reaction was stopped at a known incubation time by addition of 1 ml of 1 M sodium carbonate. The optical density at 420 nm of the mixture was measured. The β-galactosidase activity was determined using the below equation.

$$\beta\text{-galactosidase Activity} = \frac{1000 \times OD_{420} \times D \times 2.5 \times 3.5}{T \times 4.5 \times V \times OD_{560}}$$

Where:

D= dilution factor of the lysate

2.5= the coefficient factor used to convert absorbance at 650 nm into mg dry mass bacteria

3.5= total reaction volume (ml)

T = incubation time for yellow colour to develop in the reaction (min)

4.5= the molar extinction coefficient for ortho-nitrophenyl at 420 nm

V= volume of lysate (ml)

OD_{650}= the absorbance of the culture at lysis point measured at 650 nm

OD_{420}= the absorbance of the reaction at 420 nm

2.17 Growth and β-galactosidase assays in response to the addition of nitrite and nitrate

Purified transformants with *hcp::lacZ* fused to pRW50 were grown anaerobically in MS medium supplemented with LB (5%), glycerol (0.4%), TMAO (20 mM), sodium fumarate (20 mM) and 35 μg ml^{-1} Tetracycline, in two 100 mlflasks filled with 100 ml medium. The optical density at 650 nm was monitored until it had reached 0.2, then 2.5 mM nitrite or 5 mM nitrate added to one flask and the other was left as an unsupplemented control. Each culture then distributed equally by using a magnetic stirrer, but briefly to avoid aeration. Cultures were incubated statically at 37ºC. The optical density at 650 nm was monitored for at least 2 hours following the nitrite pulse, and samples (1 ml) were taken after 2 hours or 4 hours and lysed to be assayed for β-galactosidase activity.

2.18 Growth of bacteria used for releasing aconitase and fumarase activity

For aconitase and fumarase assays, single colony of required strains was inoculated in 20 ml of LB and incubated at 37ºC aerobically at 150 cycles per minute in a rotary shaker for 5-8 hours. After incubation, the 20 ml of culture was used to inoculate 250 ml minimal salts medium in a 250 ml flask that had been filled to the neck. Then the flask was also supplemented with 40 mM glycerol as the main source of carbon and energy and 40 mM fumarate as the terminal electron acceptor (2 flasks per strain). The cultures then were grown anaerobically overnight at 37ºC. The overnight cultures were used to inoculate 1 L of minimal salts medium (two flasks for each strain). These flasks were then grown anaerobically at 37ºC. The optical density at 650 nm was monitored until it had reached 0.2-0.25. Each culture was pulsed with 1.5 μM NOSW by using syringe every 30 minutes. The needle was inserted under the surface of the culture to avoid oxygen exposure and to distribute the NOSW equally

in the culture. A large magnetic stirrer was used very briefly to avoid aeration. The optical density at 650 nm was measured every hour for 5 hours.

2.19 Cell extraction and preparation of cell lysate

Most of the cells were harvested by centrifugation at 10,000 rmp, 4ºC for 10 minutes. Cells obtained from harvesting were homogenised in 50 mM phosphate buffer at pH 7.4, and then the cell suspension was centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and centrifuged at 15,000 rmp for another 10 minutes. Harvested cells were again homogenised in 50 mM phosphate buffer at pH 7.4 and the cells were sonicated for 8 minutes (30 seconds on and 30 seconds off) intervals. The sonicated cells were centrifuged at 15,000 rmp for 15 minutes and then the supernatant was centrifuged at 39,000 rmp for 1 hour. The soluble supernatant was then used for aconitase and fumarase activity.

2.20 Aconitase assay

For the aconitase assay, the Helices Omega UV-VIS spectrophotometer from Thermo Scientific was used. The recording time for each assay was 3 minutes. The assay mixture for aconitase activity was 928 µl of assay buffer (50 mM Tris-Hcl, 0.6 mM $MnCl_2$, pH 7.4), 30 µl of 1 M sodium citrate and 2 µl of 0.1 M $NADP^+$ (was made freshly and kept in ice), then was added to 1 ml cuvette. This mixture was used to zero the spectrophotometer and measured the absorbance of $NADP^+$ at 340 nm at 25ºC. The reaction was started by the addition of 40 µl of supernatant containing the enzyme (incubated on ice). For each strain, the activity of aconitase was measured in triplicate. The absorbance increased over time due to the reaction catalysed by aconitase. At least two independent experiments were completed unless otherwise specified. Standard deviations of repeats were represented by error bars.

Units/ml. enzyme = (ΔA_{340nm}/min Test -ΔA_{340nm}/min Blank)*(df)*(1)/(v)*(6.22)

Where

df= dilution factor

1= total volume (ml)

V= Volume (ml) of enzyme used

6.22= Millimolar extinction coefficient of NADPH at 340 nm

2.21 Fumarase assay

The same spectrophotometer was used for fumarase as for the aconitase assay. The recording time for each assay was set at 3 minutes, and then fumarase activity in soluble supernatant extracts was measured. The assay mixture (1 ml) contained 845 µl of phosphate buffer pH 7.4 (incubated at room temperature),), 8 µl sodium fumarate, 50 µl of 40 $mg.ml^{-1}$ NAD^{+} (freshly made and incubated on ice), 50 µl of 1 M sodium glutamate and 7 µl of 0.3 $mg.ml^{-1}$ glutamate oxaloacetate aminotransferase (incubated on ice). This mixture was used to zero the spectrophotometer and the activity of the fumarase was measured by following the absorbance of NADH at 340 nm at room temperature. The reaction was started by adding 40 µl of supernatant extract. The absorbance increased due to the reaction catalysed by fumarase. For each strain, the activity of fumarase was measured in triplicate. One unit of fumarase activity corresponds to 1 µmol NADH formed in a minute. The activity of the fumarase was calculated in unit per ml from the following formula:

Activity (U/ml)= [ΔA/min*1000}/{6.22*Volume of the supernatant in reaction mixture (µl) . Where:

6.22 = Millimolar extinction coefficient of NADH at 340 nm

2.22 Folin–phenol assay

To determine protein concentration for above assays, the same supernatant for aconitase and fumarase was used. The supernatants were diluted 1 to 100 with distilled water. A set of clean dry test tubes were used. The distilled water (0.6 ml) was added to the first test tube and was labelled as a blank, and bovine serum albumin (BSA) at 1 mg ml^{-1} was used as a standard. The diluted supernatant (50 µl) and the same volume of BSA of were added to the test tubes that containing 550 µL of distilled water. Folin B (3 ml) was added to each sample repeated in triplicate and then were mixed by vortexing. The samples were incubated at room temperature for 10 minutes. After incubation, the tubes were mixed thoroughly again and 0.3 ml of Folin phenol reagent was added and vortexed. The tubes were again incubated at room temperature for 30 minutes to allow the blue colour to develop. A blank tube was used to zero the spectrophotometer, and the optical density for each sample was measured at 750 nm. The amount of protein was calculated in mg per ml from the formula below:

Protein concentration ($mg.ml^{-1}$)= [Average OD_{750nm} of each sample*dilution factor]/OD_{750nm} of BSA

2.23 DNA techniques

2.23.1 Polymerase chain reaction (PCR)

Polymerase chain reaction is a method used to replicate a piece of DNA between two short oligonucleotides primers using a thermostable DNA polymerase. For this study, Alta Biosciences (University of Birmingham) provided the primers that were used to amplify desired gene, and are listed in Table 2.4. All primers that need to be prepared for PCR were supplied as a solid dry. Before using the primers, each one was resuspended in sterile distilled

water to a final concentration of 100 µM. Each PCR reaction was 49 µl containing 2 µl of each primer at 10 µM concentrations, 5 µl of genomic DNA was used as template and 45 µl of supermix buffer. For colony PCR, a single colony was picked from an agar plate and resuspended in 100 µl distilled water. This suspension was heated to 95°C for 10 minutes, centrifuged for 2 minutes, and then 5 µl of this suspension was used as template. The PCR reaction consists of three stages. The first stage uses high temperature to melt the template DNA and separate the two strands (denaturation). The second stage involves cooling the reaction to a optimal temperature for primer binding (annealing). Thirdly, the temperature is increased to the optimal temperature for DNA polymerase activity and the template strand is replicated (elongation). For invitrogen supermix polymerase, the elongation temperature was 72°C. A typical programme was used for the expected size of the desired nucleotide sequence and the annealing temperature of the specific primers (Table 2.5). 1 kb of DNA was polymerised per minute and the annealing temperature of each primer was calculated using the formula below:

Annealing temperature= $64.9 + (G+C\ \%) \times 0.41) - 600/n$

Where:

(C+G %)= percentage of guanidine and cytosine bases

n= total length of the primer in nucleotides

In general, to complete any partial extension in each PCR protocol, a final extension step of 72ºC for 10 minutes was applied.

Table 2.5: PCR protocols used in this study

A-PCR to amplify the promoter region of *nsrR* to be cloned into pRW50

Denaturation	Annealing	Extension	No. of cycles	Hold
94ºC, 3 min	49ºC, 30 sec	72ºC, 30 sec	1	
94ºC, 30 sec	49ºC, 30 sec	72ºC, 30 sec	35	
94ºC, 30 sec	49ºC, 30 sec	72ºC, 10 min	1	4ºC

B- PCR to confirm the presence of kanamycin cassette in the place of *oxyR* gene

Denaturation	Annealing	Extension	No of cycles	Hold
94ºC, 3 min	53ºC, 30 sec	72ºC, 3 min	1	
94ºC, 30 sec	53ºC, 30 sec	72ºC, 3 min	27	
94ºC, 30 sec	53ºC, 30 sec	72ºC, 10 min	1	4ºC

C- PCR to confirm the presence of chloramphenicol in the place of *ytfE* gene

Denaturation	Annealing	Extension	No of cycle	Hold
94ºC, 3 min	50ºC, 1 min	72ºC, 3 min	1	
94ºC, 30 sec	50ºC, 3 min	72ºC, 3 min	72	
94ºC, 30 sec	50ºC, 1 min	72ºC, 10 min	1	4ºC

2.23.2 pGEM®-T Easy cloning.

pGEM®-T Easy is convenient system that used for cloning PCR products and amplification of desired genes. This vector is linearized and has 3´-terminal thymidine at both end. The T-overhangs at the insertion site increases the eligibility of PCR products by limiting the vector re-circularisation. T Easy was used following manufacturer (Promega) instructions.

2.23.3 Restriction digest of DNA

Restriction enzymes were utilized for cutting DNA fragment within a specific site. . DNA (10 to 50 µl) was mixed with one unit of restriction enzyme and the manufacturer's buffer that was appropriate for each enzyme. The restriction mixture was incubated for 2 hours at 37°C.

2.23.4 DNA purification using a QIAquick PCR purification kit

PCR products were purified using the QIAquick PCR purification kit (QIAGEN) following the manufacturer's instructions.

2.23.5 Ligation into T Easy vector

The ligation mixture with a total volume of 10 µl contained: 5 µl of 2x Rapid ligation buffer, 1 µl of pGEM-T Easy Vector, 3 µl of PCR product and 1 µl of T4 DNA Ligase. Before each use, the 2x Rapid ligation buffer was vortexed vigorously, and then the reactions were mixed by pipetting and then incubated overnight at 4º C. In separate tube, the control ligation mixture was prepared with 1 µl of sterilized water and 2 µl of control insert.

2.23.6 Agarose gel or DNA electrophoresis

Agarose gel was made by dissolving 1% of agarose (Sigma) in 1xTBE buffer, which was then boiled in a microwave until melted. Once it had cooled, the solution (100 ml) supplemented with 5 µl of 10 $m.gml^{-1}$ ethidium bromide and was poured into an electrophoresis equipment. After the gel set, it was covered with the 1x TBE buffer, then the DNA samples were mixed with sample buffer (5:1) and loaded into the wells for investigation. For estimating the size of DNA samples, a DNA ladder (1 kb or 1000 bp, New England Biolabs, Hitchin, UK) was loaded as well. A transilluminator was used to visualise the product on the gel by ultraviolet light.

Three plates of LB/ampicillin/IPTG/X-Gal were prepared for each reaction and for determining the transformation efficiency. Each tube of ligation reaction was centrifuged in order to collect the contents at the bottom of the tubes, and then a total volume of each ligation mixture (10 µl) was added to an empty sterile microcentrfuge tube (1.5 ml) on ice. To determine the transformation efficiency of the competent cells, 2-5 µl of uncut plasmid was added to sterile microcentrfuge tubes also set up on ice.

2.23.7 The transformation of ligation mixture into JM109 competent cells

The JM109 high efficiency competent cells (50-100 µl) were mixed with ligation reactions (10 µl) and the control insert DNA. All tubes were flicked gently and set on ice for 30 minutes. After incubation on ice, the cells were shocked for 50 seconds in a water bath at 42ºC and then the tubes immediately placed on ice for 2 minutes. The LB or SOC (500-1000 ml) in room temperature was added to each tube and then incubated at 37ºC for 1-2 hours. After the incubation period, the each transformation mixture (100 µl) was plated on

NA/ampicillin/IPTG/X-Gal plates, and incubated overnight at 37ºC. The positive results were white colonies that contained the recombinant plasmid with insert.

2.23.8 Small scale extraction of plasmid DNA using QIAprep spin miniprep kit

The plasmids used in this study are illustrated in Table 2.4 and figures 2.1 to 2.4. A LB (5 ml) supplemented with appropriate antibiotics was used to grow strain that carrying the desired plasmid. Plasmid DNA was then extracted from the overnight culture using the QIAprep miniprep spin kit following the manufacturer's instructions. For low copy number plasmids such as pRW50, 20 µl of elution buffer, pre-warmed to 50°C was used to elute DNA from the column.

2.23.9 Purification of DNA vector with calf intestinal alkaline phosphatase (CIAP)

In order to limit protein contamination, an equal volume of phenol-chloroform was added to the samples of DNA then the mixture was vortexed for 30 second and centrifuged at 13000 rmp for 3 minutes. After centrifugation, an aqueous layer containing DNA was formed and transferred to clean tube. For back extraction, a volume of water equal to the DNA sample was added to the phenol-chloroform. The tube was vortexed for 15 seconds and centrifuged at 13,000 rmp for 3 minutes. Another aqueous layer was produced and combined with the first extraction.

2.23.10 Ethanol precipitation of DNA vector

After preparation of aqueous layer containing DNA, a 10% volume of 3 M sodium acetate (pH 5.2) and 3 volumes of ice cold 100% ethanol were mixed with the DNA sample by inverting. The mixture was precipitated by incubation at -20ºC for 1 hour or left overnight. The solution was centrifuged at high speed (14000 rmp, 4ºC, 15 minutes) and then the supernatant was discarded. The DNA pellet was washed with 1ml of ice-cold 70% ethanol and centrifuged for 15 minutes. The supernatant was discarded and the DNA pellet was washed again but with 1 ml of ice cold 100% ethanol and centrifuged for another 15 minutes. The supernatant was discarded and the DNA pellet was dried in speed vacuum dryer for 15 minutes. The pellet was resuspended in 30 µl of sterile distilled water.

2.23.11 Extraction of DNA from agarose gels using QIAquick gel extraction kit

A sterile scalpel was used to excise DNA bands from 0.8% agarose gels under UV illumination. DNA was eluted using a QIAquick gel extraction kit following manufacturer's instructions.

2.23.12 T4 DNA ligase

T4 ligase works by formation of a phosphodiester bond between 5' phosphate group and 3' hydroxyl group of DNA fragment. Both vector DNA fragment and insert DNA that was produced from the PCR reaction were purified by QIAquick PCR purification kit following the manufacturer's instructions. The insert DNA (1 to 2 µl) was mixed with vector DNA (1 to 2 µl), ligase buffer (2µl) and ligase enzyme (1 unit) and then the volume was made up to 10 µl with distilled water. This mixture was incubated at 16ºC for 2 hours or 4ºC overnight.

2.23.13 DNA sequencing

Plasmid DNA was sequenced using the functional genomic suite at the University of Birmingham. A modified Sanger sequencing protocol was used to sequence the plasmid DNA. This system incorporates fluorescent modified terminator nucleotides into a growing strand that is then separated by capillary electrophoresis. For sequencing templates 6.8 µl of plasmid miniprep was mixed with 3.2 µl of 1 µM sequencing primer and submitted for sequencing.

2.23.14 Statistical analysis

Data were analysed for statistical significance using unpaired samples t-test using GraphPad Prism version 5. P values were calculated, and results were considered statistically significant where $p \leq 0.05$.

CHAPTER THREE-RESULTS

3.1 Relative roles of OxyR, NsrR and Fnr in sensing nitric oxide and regulating the response of *E. coli* to nitrosative stress

During its life as a pathogenic bacterium, *E. coli* is exposured to reactive oxygen species and reactive nitrogen species that are highly toxic to this bacterium. Therefore, some transcription factors play vital roles in regulating the expression of a number of genes that are required to deal with this toxicity. During aerobic growth, OxyR controls the expression of antioxidant genes in response to oxidative stress. It is activated in the presence of high levels of H_2O_2 (Storz *et al*., 1990; Mongkolsuk and Helmann, 2002; Anjem *et al.*, 2009). In both *E. coli* and *Salmonella typhimurium*, the genes that are regulated by OxyR include *katG, gorA* and *ahpCF*, which encode hydroperoxidase/catalase, glutathione reductase and alkyl hydroperoxide reductase, respectively. Expression of these genes provides protection against H_2O_2 (Christman *et al*.; 1985; Mongkolsuk *et al*., 2002).

Hausladen *et al*. (1996) found that under aerobic conditions the growth of the *oxyR* mutant strain was inhibited more than that of the *E. coli* parent strain in cultures treated with SNO-Cys. Seth *et al*. (2012) showed that endogenous S-nitrosylation of OxyR in *E. coli* occurs under anaerobic conditions during growth in the presence of nitrate. Moreover, the same group proposed that high level expression of *hcp* that encodes a hybrid cluster protein, Hcp, was dependent on S-nitrosylated OxyR to protect against S-nitrosothiols (Seth *et al.*, 2012).

NsrR is a transcriptional repressor of the Rrf2 family of regulatory proteins in a diversity of bacteria, including *Nitrosomonas europaea,* in which it was first identified

(Beaumont *et al.,* 2004). *In vitro* studies of purified NsrR protein demonstrated that it contains an iron-sulfur cluster, which is required for DNA binding activity (Tucker *et al.,* 2008, Tucker *et al.,* 2010). These clusters are believed to serve as sensors that respond when NO is available (Tucker *et al*., 2008; Yuki *et al.,* 2008). It is still unclear whether the active sensor of NO is a (4Fe-4S) centre or (2Fe-2S) centre (Tucker *et al.,* 2008). In the presence of NO, the cluster is nitrosylated and its transcriptional repression relieved to permit transcription of genes required for protection of bacteria against nitrosative stress. NsrR has been demonstrated to negatively regulate the expression of many genes involved in the nitrosative stress response: *ytfE*; *hmp*; *nrfA*; *hcp-hcr; ygbA*; and *yeaR-yoaG* (Bodenmiller and Spiro, 2006, Filenko *et al.,* 2007).

In addition to OxyR and NsrR, the regulator of fumarate and nitrate reduction, FNR, is able to detect directly the presence or absence of O_2 by a non-haem Fe-S cluster (Becker *et al*., 1996; Jordan *et al*., 1997). The [Fe-S] is essential for protein dimerization and binding to DNA. Under aerobic conditions, the [4Fe-4S] of FNR dissociates converting the protein into inactive monomers that are unable to bind DNA (Lazazzera *et al*., 1996; Sutton *et al*., 2004a). However, under anaerobic conditions FNR exists in an active dimeric form that is able to bind specific sites in DNA (Jayaraman *et al*., 1988). Anaerobically, FNR is involved in the positive regulation of many genes implicated in nitrate and nitrite dissimilation such as the *nrf*, *nirB*, *napA* and *narG* operons (Spiro and Guest, 1987; Gennis and Stewart, 1996; Guest *et al*., 1996). Moreover, expression of the *hcp-hcr* operon is activated by FNR, especially under nitrosative stress (Filenko *et al.,* 2005) because it is also repressed by the NO sensitive repressor, NsrR (Bodenmiller and Spiro, 2006; Filenko *et al.,* 2007; Tucker *et al*., 2008). Poole *et al*. (1996) showed that *hmp-lacZ* expression in a mutant lacking *fnr* is higher than in the fnr^+ parent. Therefore, FNR represses *hmp* transcription.

Cruz-Ramos *et al.* (2002) claimed that FNR can also sense NO, which explains why *hmp* expression is enhanced during growth in the presence of high concentration of NO. But, there are some problems with these protocols. First, the experiments were accomplished before NsrR had been discovered and therefore need to be re-interpreted to distinguish between the effects of FNR and NsrR. In addition, the concentration of NO used in the experiments was so high that is sufficient to damage the [Fe-S] centres of FNR chemically. Therefore, it is important to distinguish between sensing and chemical damage.

In view of these more recent observations, the aims of this chapter are therefore first to confirm the findings of Seth *et al*. (2012). Experiments were designed to test whether OxyR activates transcription at the *hcp* promoter during aerobic and anaerobic growth in the presence and absence of the nitrate or nitrite. A new strain defective in *oxyR* was constructed, and then the effect of OxyR at the *hcp* promoter in the new strain was compared with the parent strain, RK4353. Second, the following experiments investigated the role of NsrR protein in autoregulation of its own promoter and the effect of NsrR on some genes during nitrosative stress. Third, the effects of NO on NsrR and FNR regulated genes including *hcp* and *hmp* to determine whether NO inactivates FNR or NsrR regulators.

3.2 Construction of the *oxyR* deletion in the parent strain, *E. coli* strain RK4353

Bacteriophage P1 transduction was used to transduce the *oxyR* deletion from the *E. coli* strain, MG1655Δ*oxyR::kan*, to the parent strain RK4353. The detection of the presence of the kanamycin resistance cassette (*kan*) in the place of the *oxyR* gene in strain RK4353 was confirmed by testing purified kanamycin resistant colonies by PCR with two primers, one that binds upstream and the other that binds downstream of *oxyR*. Agarose gel electrophoresis was

used to visualize the amplified PCR products, and a difference was observed between the wild type and the correct transductants. The transductants contained the correct kanamycin cassette in place of *oxyR* to produce the new strain, RK4353 Δ*oxyR*::*kan*, JCB53021, which was then available for further experiments.

3.3 Effect of the transcription factor OxyR on *hcp::lacZ* expression during aerobic growth in the presence of nitrite or nitrate

Seth *et al*. (2012) suggested that S-nitrosylation of OxyR induces the transcription of *hcp* during aerobic growth in the presence of nitrosative stress. To test whether this effect was OxyR dependent, the parental strain, RK4353 and Δ*oxyR*::*kan* strain, JCB5301, were transformed with a plasmid in which the *hcp* promoter was fused to *lacZ* in the promoter-probe vector, pRW50. Both strains were grown aerobically at 37°C in acidic minimal salts medium (pH 6.0) supplemented with 10% LB (v/v), 0.4% (v/v) glycerol, 20 mM fumarate and 20 mM TMAO in the presence or absence of sodium nitrate or sodium nitrite. After two hours, bacteria were lysed for determination of β-galactosidase activity. The β-galactosidase activity was extremely low in both the wild type and in the *oxyR* mutant (Figure 3.1). It was concluded that under aerobic conditions, S-nitrosylation of OxyR by NO generated from nitrite at pH 6.0 could not activate transcription from the *hcp* promoter.

3.4 Anaerobic growth of RK4353 and the *oxyR* mutant during nitrosative stress conditions

In order to characterize a possible role of S-nitrosylated OxyR during anaerobic growth, both the parent strain RK4353 and the JCB5301 strain were grown anaerobically at 37°C in minimal salts medium either unsupplemented or supplemented with 2.5 mM sodium nitrite

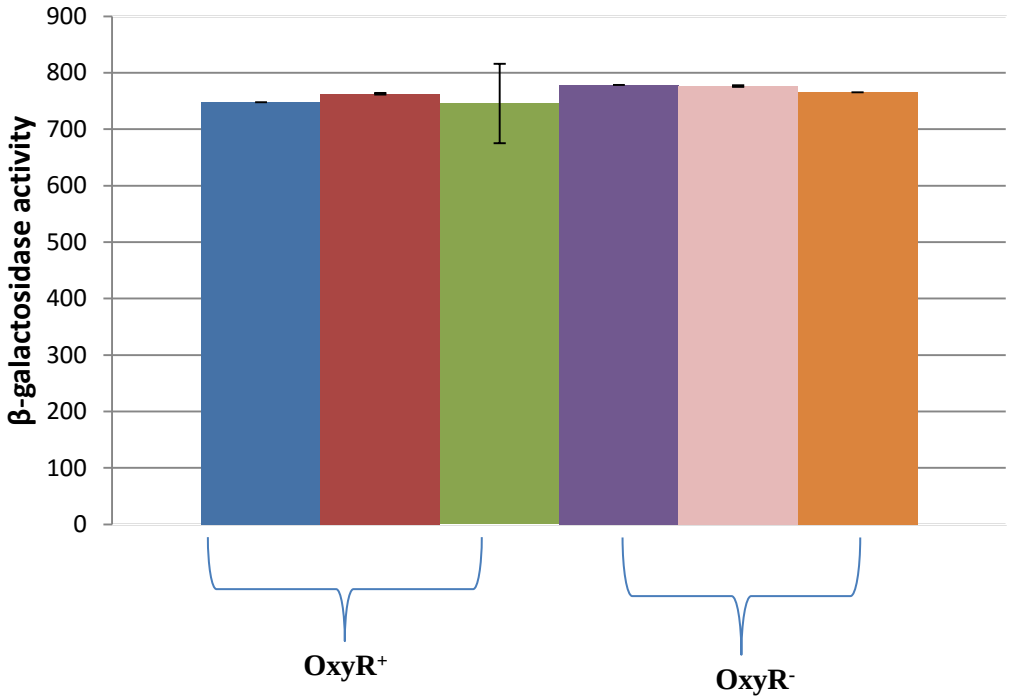


Figure 3.1: Effect of OxyR on *hcp* expression during aerobic growth

The parental strain, RK4353, and the *E. coli oxyR* derivative, JCB5301, were transformed with the plasmid in which the *hcp* promoter was fused to *lacZ* in pRW50. The purified transformants were grown aerobically in MS medium either unsupplemented (blue and purple bars) or supplemented with 0.2 mM sodium nitrite (red and pink bars) or with 2 mM sodium nitrate (green and orange bars). Samples (1 ml) were lysed and the β-galactosidase activity was assayed for each culture. Units of β-galactosidase activity in this and all subsequent experiments are nmol min^{-1} (mg dry $weight)^{-1}$. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation).

or 5 mM sodium nitrite. The optical density of each culture was monitored every hour during growth. No difference in optical density was detected between the parental strain and the *oxyR* strain in the presence and absence of nitrite and nitrate (Figure 3.2). These data suggest that deletion of *oxyR* does not significantly affect the growth of the Δ*oxyR*::*kan* strain, JBC5219, compared with that of the parent strain, RK4353.

3.5 Effect of OxyR on *hcp::lacZ* transcription during anaerobic growth in the presence of nitrite and nitrate

In order to reveal whether OxyR plays any role in the activation of transcription at the *hcp* promoter, the wild type RK4353 and the *oxyR* mutant were transformed with the *hcp* promoter fused to *lacZ* in pRW50. Bacteria were grown in minimal salts medium anaerobically in the presence or absence of sodium nitrate or sodium nitrite, they were then lysed and β-galactosidase activities were determined. The activity of the *hcp* promoter was low in the unsupplemented cultures. After 2 and 4 hours, the deletion of *oxyR* led to an increase in the anaerobic expression of *hcp* promoter in the presence of both nitrite and nitrate, and this activation was similar to that of the parent strain as there was no significant difference between both strains supplemented with nitrite ($p=0.624$) or nitrate ($p=0.612$) (Figure 3.3). These data once again confirmed that OxyR is absolutely not required for *hcp* promoter activity anaerobically in the presence of nitrite or nitrate.

3.6 Di-iron protein of *E. coli*, YtfE, is implicated in the response to oxidative stress conditions

It has been found that expression of *E. coli ytfE* gene increases anaerobically under nitrosative stress conditions and iron starvation in order to protect against nitrosative stress

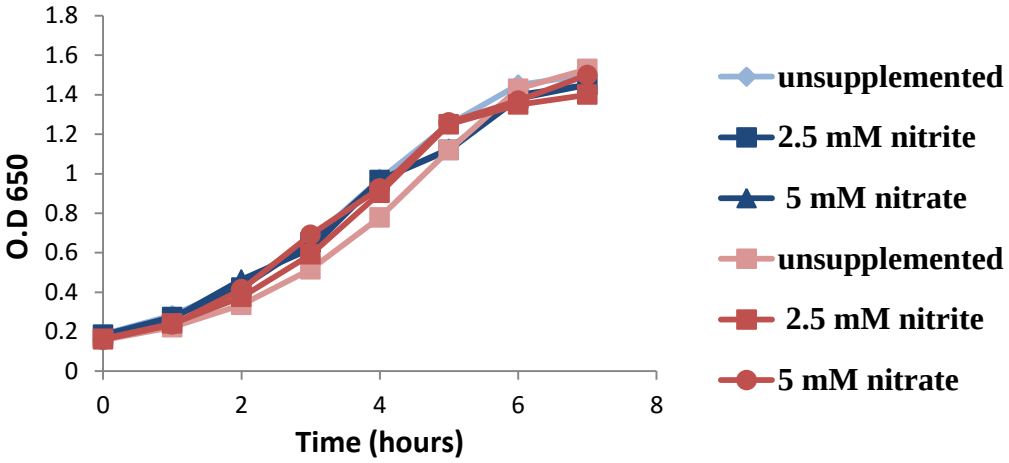


Figure 3.2: Anaerobic growth of strains RK4353 and RK4353 *ΔoxyR::kan* in the presence of nitrate or nitrite

The parental strain, RK4353 (blue line), and the *oxyR* mutant (red line), were grown anaerobically in minimal salts medium supplemented with 10% LB, 0.4% glycerol in the presence of 2.5 mM nitrite or 5 mM nitrate (dark lines) or in the absence of nitrite or nitrate (pale lines). The cultures were incubated without shaking at 37ºC and the optical density of the culture was measured every hour for 7 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

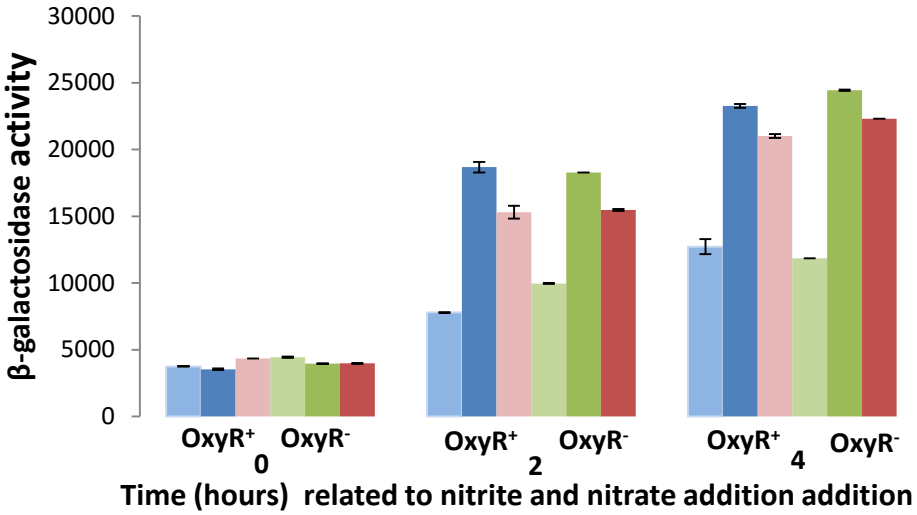


Figure 3.3: The effect of OxyR on transcription from the *hcp* promoter

The parental strain, RK4353, and the *oxyR* mutant RK4353 *ΔoxyR*::*kan*, were transformed with a plasmid in which the *hcp* promoter was fused to *lacZ* in pNF383. The cultures were grown anaerobically in MS medium supplemented with glycerol and fumarate. When the optical density had reached 0.2, two cultures were supplemented with 2.5 mM nitrite (RK4353 strain, blue bars and RK4353 Δ*oxyR::kan* strain, green bars). Other two cultures were supplemented with 5 mM nitrate (RK4353 strain, pink bars and RK4353 Δ*oxyR*::*kan* strain, red bars) while the control cultures were left unsupplemented (RK4353 strain, pale blue and RK4353 Δ*oxyR*::*kan* strain, pale green bars). The β-galactosidase activity was assayed for each culture. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation).

(Justino *et al.*, 2005, 2006). Previous studies by Justino *et al*. (2007) and Overton *et al*. (2008) had identified that YtfE also plays a crucial role in protection against oxidative stress caused by H_2O_2. To assess whether the *E. coli* YtfE protein could be involved in protection against oxidative stress, the starting strain JCB5210, constructed by Dr Claire Vine, was used which defective in all known nitrite and NO reductases including *nirBDC*, *norVW*, *nrfA* and *hmp* (Table 3.1). Three additional strains derived from the JCB5210, made by Dr Claire Vine, were used. These strains were *hcp ytfE*$^+$, JCB5250, *hcp*$^+$*ytfE*, JCB5257, and *hcp ytfE*, JCB5260 (Table 3.1).

All four strains were grown anaerobically at 37ºC in MS medium either supplemented or unsupplemented with hydrogen peroxide (H_2O_2), which was added at an optical density of 0.3 and every subsequent hour to final concentrations of 2 mM. Growth was followed for 5 hours and then compared with unsupplemented control cultures. All four strains grew equally well in the absence of 2 mM of H_2O_2 and no noticeable inhibition of growth was found in any of four strains after 5 hours of addition of H_2O_2 (Figure 3.4). These findings clearly showed that YtfE has no effective role in dealing with oxidative stress and the relief of growth inhibition by hydrogen peroxide might be due to the activation of other enzymes that confer protection against H_2O_2.

3.7 Effect of an *fnr* mutation on the adaptation of JCB5210, JCB5250 and JCB5251 to anaerobic growth in the presence or absence of nitrosative stress

The two strains used in the experiments described above (Figures 3.1, 3.2, 3.3) are highly resistant to nitrosative stress because they are *norvW*$^+$ and *nrf*$^+$ and therefore able to reduce NO to N_2O or to NH_4^+. They are also *nirB*$^+$and hence able to reduce nitrite as rapidly

Table 3.1: Strains defective in genes involved in the nitrosative stress conditions

Strain name	Genotype*	
	hcp	***ytfE***
JCB 5210	+	+
JCB5257	+	-
JCB5250	-	+
JCB5260	-	-

*Every strain is defective in all known nitrite and nitric oxide reductases *nirB nrfA norVW* and *hmp.* Other details about each strain can be found in Table 2.2.

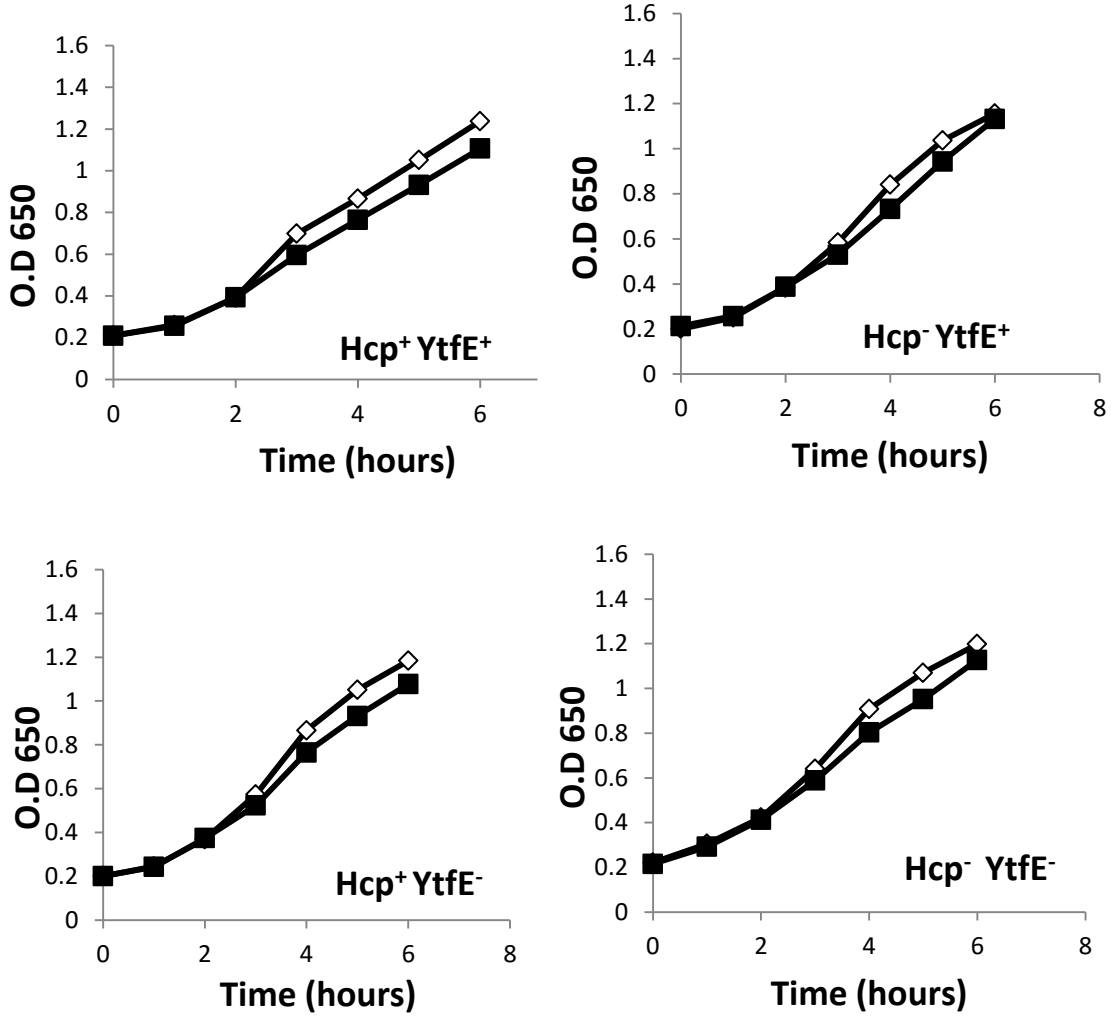


Figure 3.4: Effect of the di-iron protein, YtfE, on anaerobic growth of strains defective in all NO reductases in the presence and absence of the 2 mM H_2O_2

The set of four strains *hcp*$^+$*ytfE*$^+$ (JCB5210); *hcp ytfE*$^+$ (JCB5250); *hcp*$^+$ *ytfE* (JCB5257); and *hcp ytfE* (JCB5260) deleted for *nirBD*, *nrfAB*, *norV* and *hmp* genes, were grown under anaerobic conditions in MS at 37ºC without shaking in the absence of H_2O_2 (open symbols) or in the presence of 2 mM H_2O_2 (filled symbols), which was added every hour. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

as it is formed to NH_4. This would minimise the rate of production of NO in the cytoplasm. In order to investigate whether a transcription at the *hcp* promoter is regulated by FNR independently of OxyR for protecting *E. coli* against NO toxicity, it would therefore be necessary to use strains that are much more sensitive to nitrosative stress. The *nirBDC nrfAB norV hmp hcp* strain, JCB5250 and *nirBDC nrfAB norV hmp* strain, JCB5210 were therefore used in subsequent experiments. Note that these strains retain a functional $oxyR^+$ gene. To this end, the growth of both strains was compared to the growth of the *nirBDC nrfAB norV hmp fnr* strain, JCB5251 that lacks the *fnr* gene. All three strains were grown anaerobically in the minimal medium at 37ºC without shaking in the presence and absence of 0.1 μM NO, which was added every 30 minute. The optical density was measured every hour for 6 hours. Strain JCB5210 grew well in the presence and absence of NO. However, the growth of JCB5250 was totally inhibited when NO was introduced. Moreover, the growth of JCB5251 was also inhibited by additions of 0.1 μM NO every 30 minutes (Figure 3.5). Thus, it can be concluded FNR is the key player in protection under the conditions tested.

3.8 The effect of an *fnr* mutation on *hcp::lacZ* expression

Several studies reported that transcription at the *hcp* promoter is mainly under the control of FNR (Kim *et al.,* 2003; Filenko *et al.,* 2005 and Chismon *et al*., 2010). Extensive microarray studies found that the *hcp* promoter also contains a DNA binding site for the nitrite/nitrate regulators NarL and NarP (Filenko *et al*., 2005; Chismon *et al*., 2010). Moreover, Filenko *et al*. (2007) demonstrated that the nitrite-sensitive repressor, NsrR, regulates the expression of the *hcp-hcr* operon. Almeida *et al.* (2006) confirmed experimentally that expression of *hcp* under aerobic and anaerobic conditions in the presence of H_2O_2 was OxyR dependent. Seth *et al*. (2012) also claimed that the *hcp* expression was

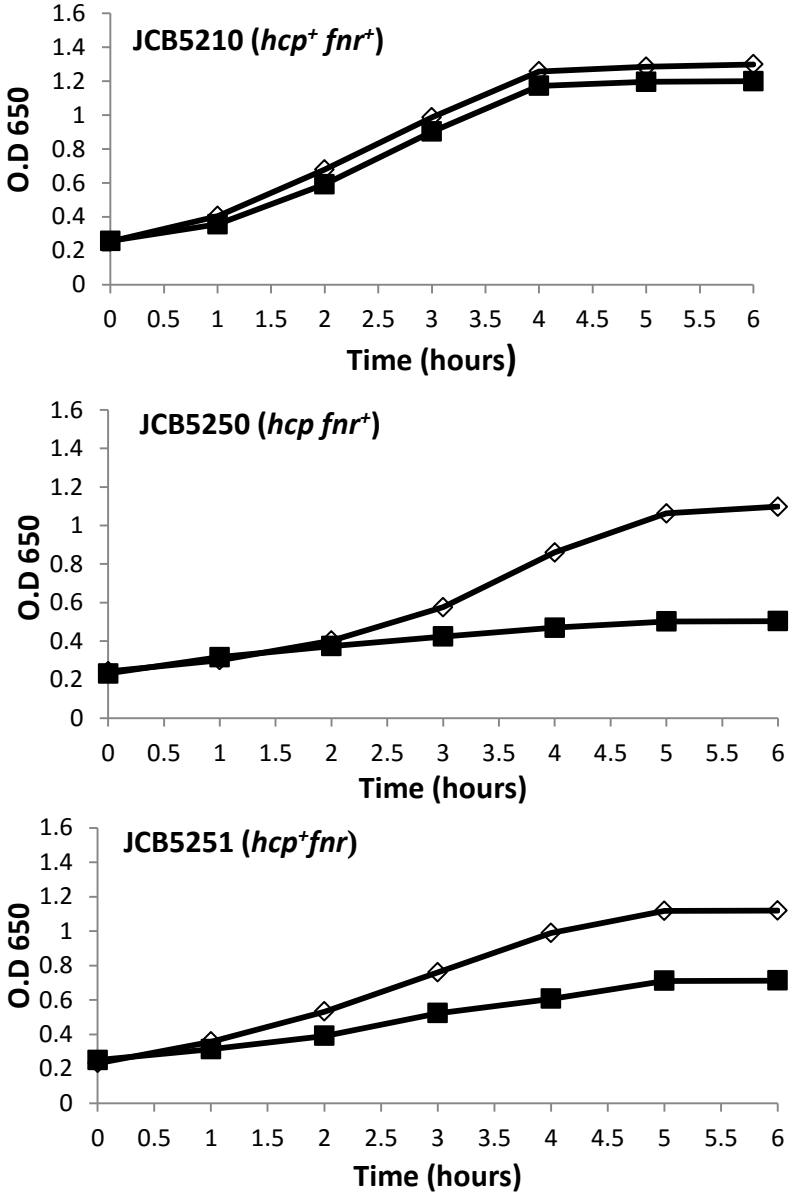


Figure 3.5: Adaptation of *E. coli* strains to anaerobic growth in the presence or absence of nitrosative stress

The parent strain, *nirBDC nrfA norV hmp*, JCB5210, the *nirBDC nrfA norV hmp hcp* strain, JCB5250, and the *nirBDC nrfA norV hmp fnr* strain, JCB5251, were grown anaerobically in minimal medium at 37ºC in the presence of 0.1 µM of NO (filled symbols) or in the absence of NO (open symbols), which was added every 30 minutes for 6 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

induced both aerobically and anaerobically in the presence of nitrite and nitrate by OxyR. However, none of these previous studies resolved whether the effects reported were due to direct regulation of *hcp* transcription by OxyR or NarL, or due to secondary consequences of their absence. For example, in the absence of OxyR, it was possible that FNR is less active due to oxidative damage and that loss of *hcp* transcription is due to the absence of active FNR. The next series of experiments were designed to test this possibility. Multiple attempts to construct *oxyR* derivatives of *nirBDC nrfAB norV hmp fnr* strain, JCB5111, and *nirBDC nrfAB norV hmp hcp fnr* strain, JCB5112 that lacks FNR were unsuccessful even when anaerobic conditions and addition of glucose were used in P1 mediated transduction experiments.

In order to investigate whether induction of the *hcp* promoter is due FNR or OxyR, the parent strain, JCB5210, and its derivative *nirBDC nrfAB norV hmp fnr* strain, JCB5251, were therefore transformed with the *hcp::lacZ* plasmid. The strains were grown anaerobically in the presence and absence of 0.2 mM nitrite. The optical density for each culture was measured every hour for 2 hours. Cultures samples (1 ml) were then lysed just before nitrite addition and 2 hours after nitrite addition and assayed for β-galactosidase activity. As expected, only a low activity was detected in strain JCB5251 that lacks FNR in the presence or absence of nitrite (Figure 3.6). However the activity of the *hcp* promoter in the FNR^+ strain JCB5210 increased significantly in the presence of nitrite compared to the FNR^- strain JCB5251 ($p=0.0003$). These data suggest that FNR is essential for the activation of *hcp* transcription while OxyR, which is functional in the strains used was unable to replace the requirement for FNR in *phcp* expression. It was also interesting to investigate whether NO has the same effect as nitrite on *hcp* transcription. Transformants were grown anaerobically in the presence or absence of 2 μM NO for strain JCB5210 and 0.1 μM of NO for strain JCB5251.

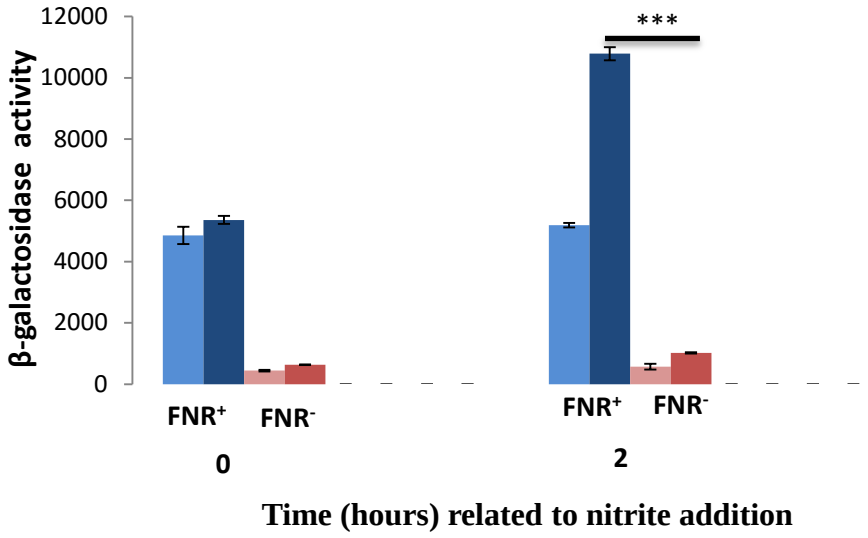


Figure 3.6: The effect of FNR on anaerobic nitrite transcription activation at the *hcp* promoter

The *nirBDC nrfAB norV hmp* parent strain, JCB5210 (blue), and *nirBDC nrfAB norV hmp fnr* strain, JCB5251 (red), were transformed with *hcp* promoter fused to *lacZ* in pRW50. The cultures were grown anaerobically in MS medium that was unsupplemented (pale bars), or supplemented with 0.2 mM sodium nitrite (dark bars). The β-galactosidase activity was assayed for each culture when their optical density had reached 0.2. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation).*,**,*** represents significance ($P<0.05$), ($P<0.01$) and ($P<0.005$) respectively .

The β-galactosidase activity of the cultures were determined after growth for 4 hours. The β-galactosidase activity in the *fnr*$^{+}$ strain increased drastically in response to NO. In contrast, in the *fnr* mutant the NO mediated activity of the *hcp* promoter was almost completely abolished (Figure 3.7). Again, these results confirmed that OxyR is not responsible for the activation of transcription at the *hcp* promoter, but FNR.

3.9 Construction of the *Eco*RI/*Hin*dIII *nsrR* promoter fragment by polymerase chain reaction and cloning of this promoter into pRW50

In order to investigate whether NsrR protein autoregulates its own promoter and the effect of NsrR on some genes during nitrosative stress, a new plasmid had to be constructed. The *nsrR* promoter region consisting of 300 pb was amplified from MG1655 chromosomal template using PCR (Figure 3.8). Two primers introduced an *Eco*RI site and a *Hind*III site at the 5' and 3' end of the fragment, respectively (Table 2.3). DNA products were purified using agarose gel electrophoresis on a 1 % agarose gel and extracted using a Qiagen gel extraction kit following the manufacturer's instructions. Purified PCR products were digested with *Eco*RI and *Hin*dIII, purified using the QIAGEN PCR purification kit and quantified by gel electrophoresis on a 1% agarose gel. The PCR product was also ligated directly into a T-easy (Promega) vector and transformed into supplied super competent cells following manufacture's structures.

Vector DNA was isolated from a transformant using a Qiagen mini-prep kit, digested with *Eco*RI and *Hin*dIII and then treated with alkaline phosphatase. The dephosphorylated vector was purified by phenol/chloroform extraction and ethanol precipitation. The DNA insert was ligated into *Eco*RI-*Hin*dIII digested vector using T4 DNA ligase and incubated

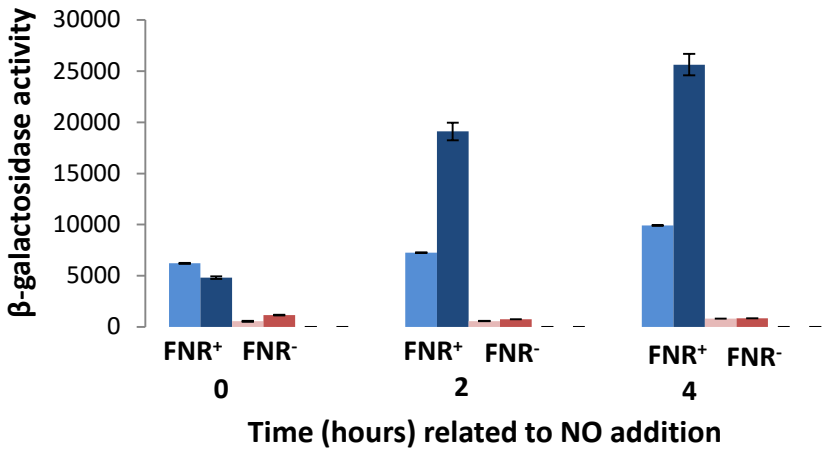


Figure 3.7: The effect of FNR on anaerobic NO transcription activation at the *hcp* promoter

The *nirBDC nrfAB norV hmp* parent strain, JCB5210 (blue), and *nirBDC nrfAB norV hmp fnr* strain, JCB5251 (red), were transformed with *hcp* promoter fused to *lacZ* in pRW50. The cultures were grown anaerobically in MS medium in the absence of NO (pale bars), or in the presence of the 2 µM NO the *fnr*$^{+}$ strain (dark blue bars) or 0.1 µM NO for the *fnr* strain (dark red bars). The β-galactosidase activity was assayed for each culture when their optical density had reached 0.2. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation).

EcoRI

GAATTCCTGCGCGACCCGTTCGACGCGTAATTCTGGTACGCCTGGCAGATAT

TTTGCCTCCGGGCGAACAGTGTGATACATTGCTGTGTCGGGTAAGCCATTACGCT

-35 **-10** **+1**

ATCCGACACAGTGTTAAATCCTCGCTTTTTTCCTTCCCCGAACTGAAATAAATTAG

CGACACAGCTTGTGGCTGGTTTATCATCAATATAAATGTATTTTTTCCCGATTTCC

CTTTTGAGGTTGATGTGCAGTTAACGAGTTTCACTGATTACGGATTACGTGCGCTG

ATCTACATGGCGTCATTGCCAGGGGCGAAGCTT

HindII

Figure 3.8: The *nsrR* promoter sequence

The figure shows the *nsrR* promoter sequence that was amplified by PCR. *Eco*RI and *Hind*III sites are blue coloured. Predicted promoter -35 and -10 elements and the transcription start site +1 are coloured red

16°C for 2 hours or 4°C overnight. A aliquot (10 μl) of the ligation mixture was used to transform competent cells prepared by the rubidium chloride method as previously described and selected on MacConkey-lactose agar supplemented with tetracycline. Candidates were screened for the presence of an insert by both *EcoRI*/*Hin*dIII digestion and PCR using primers that flank the cloning linker. The functional genomics centre operated by the University of Birmingham sequenced candidates that showed a positive result for an insert. The pBKB1 plasmid was used for the following series of experiments.

3.10 Lack of autoregulation of the *nsrR* promoter by NsrR

Expression of genes for many transcription factors is autoregulated by the gene product. If the expression of the *nsrR* gene in response to nitrite is NsrR dependent, the β-galactosidase activities should be higher in cultures of the parental strain, RK4353, after growth in the presence than in the absence of nitrite. To test this prediction, the *nsrR* promoter fragment was fused to *lacZ* into the plasmid, pRW50, as described above, then was transformed into the parent strain, RK4353. Purified transformants were grown both aerobically and anaerobically at 37ºC in the minimal salts medium in the presence and absence of 2.5 mM nitrite until the optical density had reached 0.2 at 650 nm.

The β-galactosidase activities of the lysed bacteria were determined. During aerobic growth, a very low basal level of transcription from the *nsrR* promoter in the parent strain was detected (Figure 3.9). Moreover, the activity of the *nsrR* promoter in the parental strain grown anaerobically in the absence of nitrite was also low and it did not change even when the medium was supplemented with 2.5 mM nitrite, the difference was statistically not significant ($p= 0.605$) (Figure 3.9). These data revealed that only a low level of *nsrR* promoter activity was detected under either aerobic or anaerobic conditions.

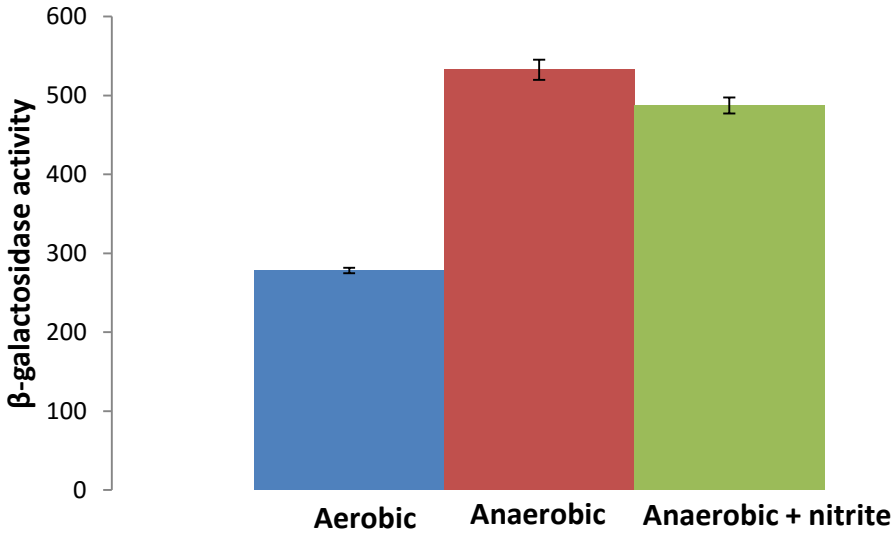


Figure 3.9: The effect of NsrR on transcription from the *nsrR* promoter in RK4353 strain

The parental strain, RK4353, was transformed with the plasmid pBKB1 and grown aerobically (blue bar) for 2 hours, anaerobically in the absence of nitrite (red bar) and anaerobically in the presence of nitrite (green bar) for 4 hours, and then β-galactosidase activities of lysed cultures were assayed. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation).

3.11 Effect of an *nsrR* mutation on the expression of *PnsrR::lacZ* during anaerobic growth in *nsrR* mutant strain and wild type

To further investigate whether the NsrR protein regulates transcription at the *nsrR* promoter, it was necessary to use the strain that lacks the *nsrR* gene. The RK4535 *ΔnsrR* strain, JCB5222 and also its parental strain, RK4353 were transformed with plasmid pBKB1, and then were grown anaerobically at 37ºC in MS supplemented with glycerol and fumarate, in the presence or absence of 2.5 mM sodium nitrite. The optical density at 650 nm was measured every hour for 8 hours. Samples (1 ml) after 4 hours were lysed for β-galactosidase activity. Both strains grew well in the presence and absence of sodium nitrite (Figure 3.10, A), but The β-galactosidase activity of the JCB5222 strain was not significantly higher or lower than in the parent RK4535 strain (p= 0.689) (Figure 3.10, B). No evidence was obtained to suggest that the *nsrR* promoter is autoregulated by the NsrR protein.

3.12 Construction of a new *nsrR* mutation in cured quadruple mutant strains, JCB 5249, and its derivative strain, JCB5253

Data presented so far indicate that Hcp is essential for the removal of low concentrations of NO from the cytoplasm (Wang *et al*., 2016, in submission). Although this was consistent with Hcp itself being the high affinity NO reductase, it did not exclude the alternative possible explanation that Hcp might be essential to prevent complete inactivation of NsrR, which in turn is essential to activate the expression of an unidentified gene encoding the NO reductase. To exclude this possibility, an isogenic set of mutants was required in which either NsrR or Hcp or both of them were inactivated. Bacteriophage P1 transduction

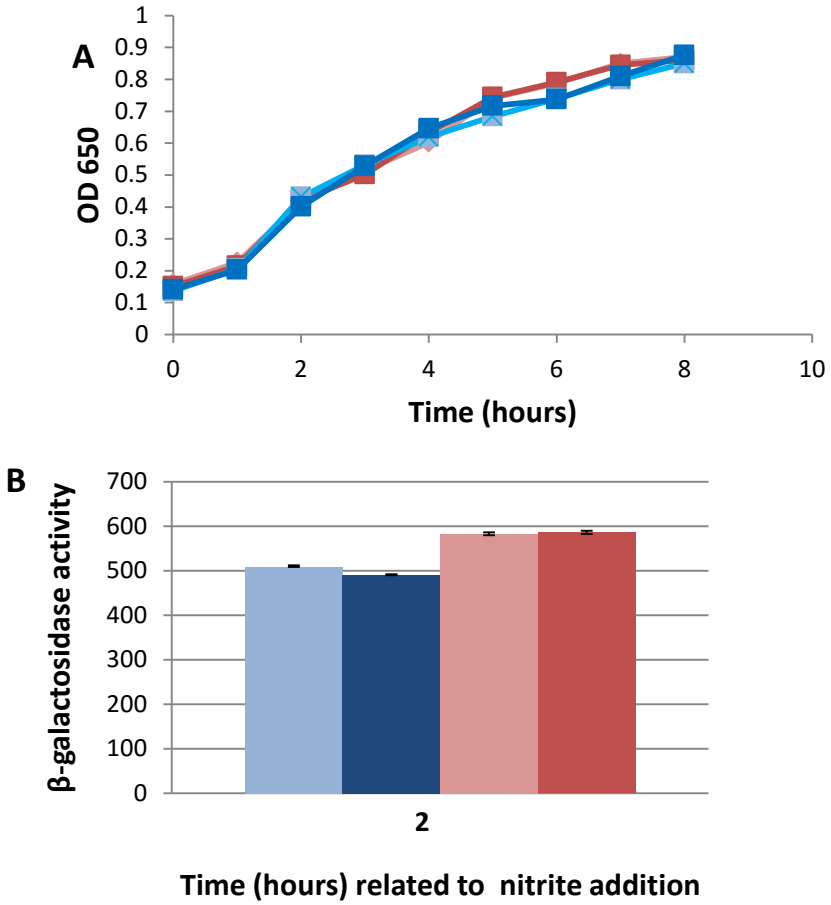


Figure 3.10: The effect of the NsrR protein on anaerobic growth and on the activity of the *nsrR* promoter in NsrR$^+$ and NsrR$^-$ strains

The parental strain, RK4353 (blue), and RK4353Δ*nsrR*::*cat* (red) were transformed with plasmid pBKB1 in which the *nsrR* promoter fused to *lacZ* in pRW50. The cultures were grown anaerobically in MS medium that was unsupplemented (pale blue, pale red), or supplemented with 2.5 mM sodium nitrite when the O.D had reached to 0.2 (dark blue, dark red). The β-galactosidase activity was assayed for each culture when their optical density had reached 0.4. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation).

has been used to transfer the *nsrR* mutation from RK4353 Δ *nsrR*, JCB5222 into *hcp*$^{+}$strain, JCB5249 to generate an *hcp*$^{+}$*nsrR* strain, JCB5218 and also into the *hcp* mutant strain, JCB5253, to generate *hcp nsrR* strain JCB5258. The presence of the kanamycin resistance cassette (*kan*) in place of the *nsrR* gene was confirmed by testing purified colonies from kanamycin resistant transductants by PCR with two primers that bind upstream or downstream of *nsrR*. Agarose gel electrophoresis was used to visualize the amplified PCR products. The transductants contained the correct kanamycin cassette in the place of the *nsrR* gene to confirm that both of the new strains were correct and available for further experiments.

3.13 The effect of the *nsrR* mutation on growth of strains that are sensitive to nitrosative stress

To investigate whether NsrR fulfills an indirect role in protection against nitrosative stress, both the *nsrR*$^{+}$ strain, JCB5249, and its *nsrR* derivative, JCB5218 that are defective in all NO reductases were grown in MS at 37ºC in the presence and absence of 2.5 mM nitrite or with repeated additions of NOSW at concentrations of 2 or 5 μM NO. The optical density was measured every hour for 6 hours. Both strains, JCB5249 and JCB5218, grew well in the presence of nitrite or 2 μM of NO, but both were severely inhibited by 5 μM of NO (Figure 3.11, A, B). These experiments revealed that loss of the NsrR did not result in any detectable increase or decrease in sensitivity to nitrosative stress. Clearly, NsrR is not essential for stress resistance in strains when its NsrR is active.

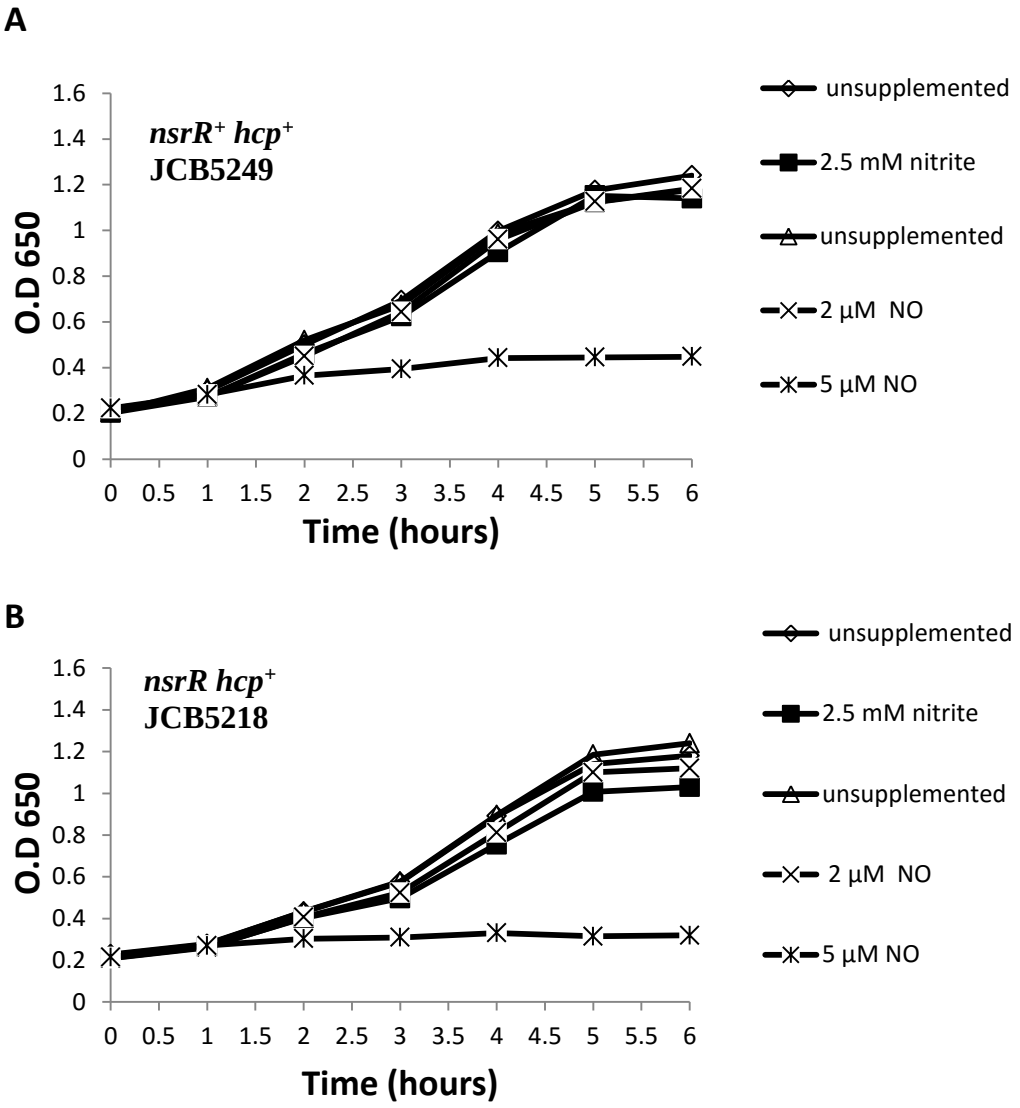


Figure 3.11: Growth phenotype of the *nsrR*$^+$strain, JCB5249, and its *nsrR* derivative strain, JCB5218, in MS unsupplemented or supplemented with nitrite or NO

The *nsrR*$^+$ *hcp*$^+$ strain, JCB5249, and its *nsrR hcp*$^+$derivative, JCB5218, were grown anaerobically in MS at 37ºC in the presence of 2.5 of nitrite or 2 or 5 µM NO and in the absence of nitrite or NO. The optical density for each strain was measured every hour for 6 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

3.14 The effect of an *hcp* mutation on strains that retain or lack NsrR

The two strains used in this experiment are sensitive to nitrosative stress because they are NirB$^-$, Hmp$^-$, NorvW $^-$and Nrf $^-$and therefore unable to reduce NO to N_2O or NH_4^+. They are also Hcp$^-$, and hence unable to reduce NO as rapidly as it is formed to N_2O. This would

increase the accumulation of NO in the cytoplasm. In order to investigate the effect of an *hcp* mutation in these strains that lack or retain NsrR, the *nsrR*$^+$*hcp* strain, JCB5253, and its derivative *nsrR hcp* strain, JCB5258, were grown in MS at 37ºC in the presence and absence of 2.5 mM nitrite or with repeated additions of NOSW at concentrations of 2 or 5 μM NO. Then the optical density was measured every hour for 6 hours.

The *nsrR*$^+$ *hcp* strain, JCB5253, and derivative mutated in *nsrR* and *hcp* were equally sensitive to 2.5 mM nitrite or 5 μM NO (Figure 3.12, A, B) and much more sensitive than the corresponding *hcp*$^+$strains (Figure 3.11, A, B), indicating that the phenotypes of the *nsrR* mutant strain were identical to those of the corresponding *nsrR*$^+$ strains. This confirmed that loss of NsrR repressor function is not required for growth under stress. This also indicates that the phenotypes of the *hcp* mutant are not indirect effects of the loss of a functional NsrR protein

3.15 The effect of nitrosative stress and Hcp on constitutive expression of promoters activated or repressed by FNR

In order to further investigate whether NsrR plays an indirect role in the activation of expression of another NO reductase gene, the two *nsrR* derivatives strains, JCB5218, in which Hcp is functional and JCB5258 in which Hcp is absent were transformed with the *hcp::lacZ*

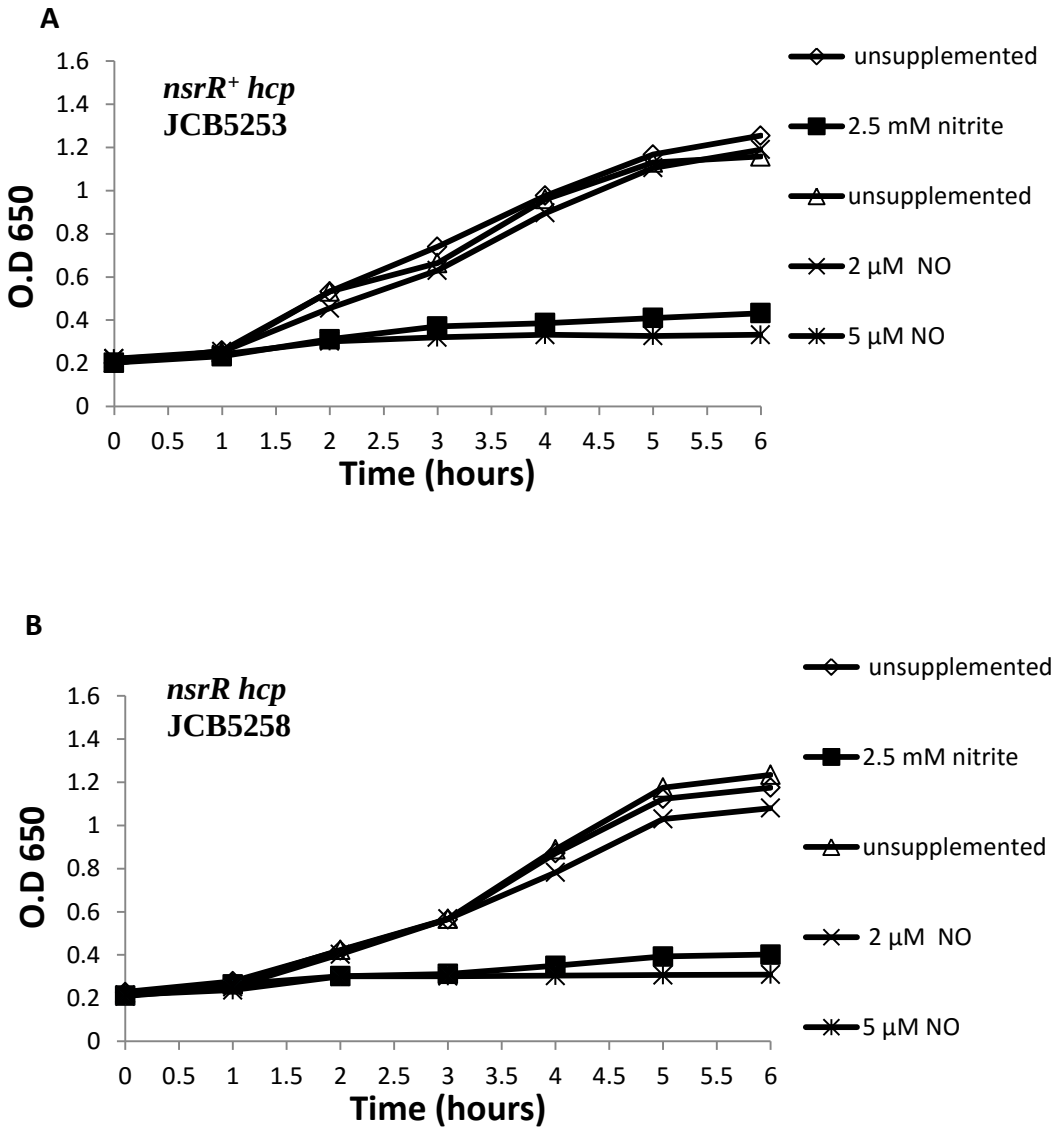


Figure 3.12: Growth phenotype of the *nsrR*$^+$strain, JCB5253, and its *nsrR* derivative strain, JCB5258, in the presence of nitrite or NO

The *nsrR*$^+$ *hcp* strain, JCB5253, and its *nsrR hcp* derivative strain, JCB5258, were grown anaerobically in MS at 37ºC in the presence of 2.5 of nitrite or 2 or 5 µM NO and in the absence of nitrite or NO. The optical density for each strain was measured every hour for 6 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

promoter fusion (activated by FNR but repressed by NsrR) and *hmp::lacZ* promoter fusion (repressed by both FNR and NsrR).Both transformants were grown anaerobically in MS at 37ºC in the presence or absence of low concentrations of sodium nitrite (0.1 mM), and then they were lysed and assayed for β-galactosidase activity. The β-galactosidase expression for both promoters (*hcp::lacZ*) (*hmp::lacZ*)was not significantly different in the *nsrR hcp*$^{+}$ strain and *nsrR hcp* strain (p= 0.4508) (P=0.4305) respectively.

Therefore, derepression of *hcp::lacZ* expression indicated that FNR was functional. However, derepression of NsrR-regulated genes failed to replace the requirement of a functional Hcp protein for resistance to nitrosative stress (Figure 3.13).

3.16 Evidence that Fnr is not a physiologically relevant NO sensor

Cruz-Ramos *et al.* (2002) demonstrated that during anaerobic growth, NO reacts with the iron-sulfur centre of FNR, generating a dinitrosyl-iron complex, which in turn causes its inactivations. If the accumulation of NO in the *E. coli* cytoplasm during nitrite reduction can damage FNR, the activity of the *hcp* promoter in the *hcp::lacZ* transformants will be much lower than that of the *hmp::lacZ* transformants. In contrast, low concentrations of NO will inactivate NsrR but have little effect on FNR, so the activity of both promoters will go up.

To confirm this prediction, the parental strain, RK4353 and its *nsrR* mutant, JCB5222, were transformed with the *hcp::lacZ* promoter fusion (which is activated by FNR but repressed by NsrR) and the *hmp::lacZ* promoter fusion (repressed by both FNR and NsrR). The transformants were grown anaerobically at 37ºC in MS in the presence and absence of the 2.5 mM nitrite. Samples (1 ml) were lysed just before addition of nitrite and then 2 hours after

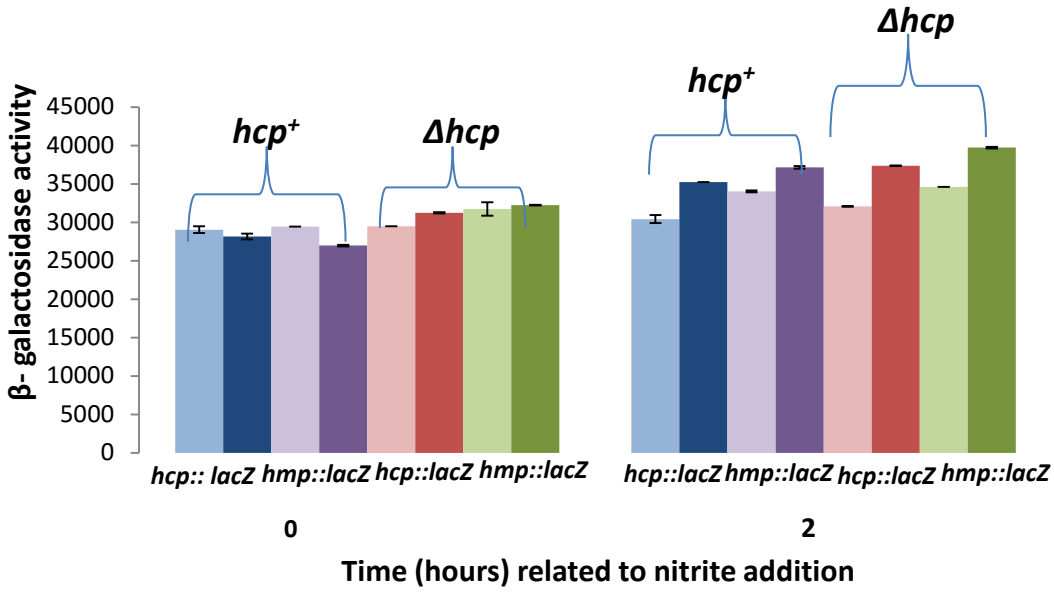


Figure 3.13: Effect of an *hcp* mutation on the constitutive expression of β-galactosidase from either the *hcp::lacZ* fusion plasmid or the *hmp::lacZ* fusion plasmid in the absence of a functional NsrR protein

The *ΔnsrR hcp*$^{+}$ strain, JCB5218, in the presence of a functional Hcp protein (blue and purple bars) and *ΔnsrR Δhcp* strain, JCB5258, in the absence of a functional Hcp protein (red and green bars), were transformed either with the *hcp::lacZ* fusion plasmid (blue and red bars) or the *hmp::lacZ* fusion plasmid (purple and green bars). The purified transformants were grown in minimal salts medium under anaerobic conditions at 37 Cº without shaking. One culture of each strain was supplemented with 0.1 mM nitrite (darker bars), and the other was unsupplemented as a control (pale bars), then cells were lysed and assayed for β-galactosidase. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation).

nitrite addition were assayed for β-galactosidase activity. As expected, the activities of the *hmp* and *hcp* promoters were much higher in the *nsrR* mutant strains both in the presence and absence of nitrite compared with the parental strain RK4353 (Figure 3.14). These data suggested that NO produced from nitrite reduction had no effect on FNR and that both the *hcp::lacZ* and *hmp::lacZ* promoters were depressed due to inactivation of NsrR, not FNR. This confirmed that FNR was still active. Hence, FNR cannot play a physiologically significant role as a sensor of NO.

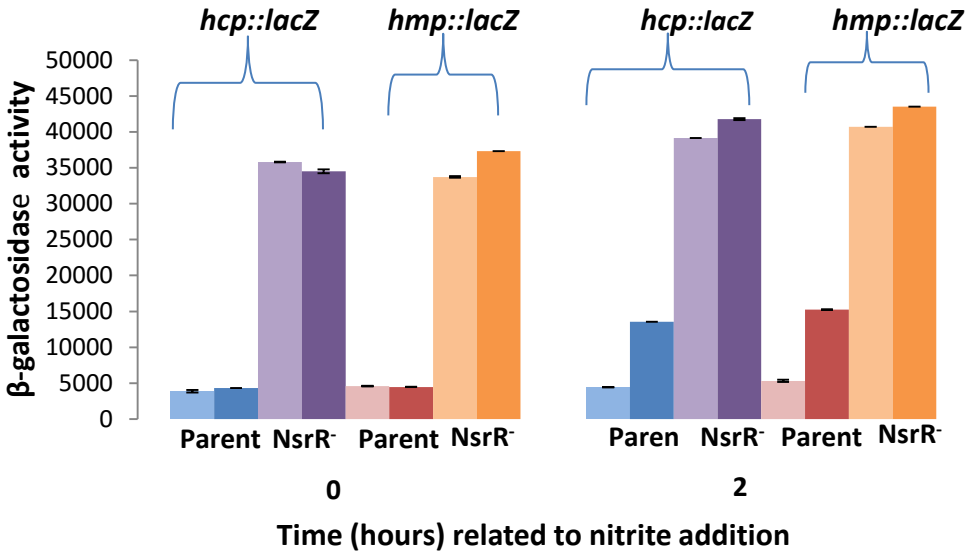


Figure 3.14: Comparison of the response of the *hcp* and *hmp* promoters to nitrite in the RK4353 and *nsrR* mutant strains

The parental strain, RK4353 (blue and red bars), and the *nsrR* mutant, JCB5222 (purple and orange bars), were transformed either with the *hcp::lacZ* fusion plasmid (blue and purple bars) or with the *hmp::lacZ* fusion plasmid (red and orange bars). The cultures were grown anaerobically in MS medium supplemented with glycerol and fumarate. Cultures were either unsupplemented (left of each coloured pair, pale bars) or supplemented with 2.5 mM nitrite (right of coloured pair, darker bars), and then samples (1 ml) were lysed and assayed for β-galactosidase activity just before nitrite addition and after 2 hour. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation).

3.17 Conclusion

By using independent reporter promoters that are repressed by NsrR, it has been confirmed that OxyR is not required for *hcp* promoter activity under anaerobic conditions in order to protect against nitrosative stress, but FNR is the key player in protection under the conditions tested. This study also confirmed that NsrR has no any significant role in regulation of its own promoter by using new constructed plasmid, pBKB. Furthermore, the deletion of the *nsrR* does not affect on growth under conditions of nitrosative stress.

This work showed that YtfE is not involved in protection against oxidative stress. Finally, exposure to concentrations of NO that are sufficient to derepress both *hcp* and *hmp* promoters were due to inactivation of NsrR, not FNR. Therefore, FNR cannot play physiologically considerable role as a sensor of NO.

CHAPTER FOUR-RESULTS

4.1 Evidence that the di-iron protein YtfE releases NO into the *E. coli* cytoplasm

The enteric pathogen *E. coli* contains a number of mechanisms that enable it to metabolize NO and other secondary products caused by NO toxicity. *E. coli* encounters NO produced from different sources. In *E. coli* there are two reduction pathways that reduce NO rapidly to ammonia. The first pathway is catalysed by the cytoplasmic nitrite reductase, NirBD, which is synthesized during anaerobic growth in the presence of a high concentration of nitrite. The alternative pathway is catalysed by the periplasmic nitrite reductase, NrfAB, which is induced during growth in the presence of a low concentration of nitrate.

A study by Smith (1983) showed that during nitrite reduction, a small amount of NO and N_2O are produced, but the rate of NO production was 100 to 1000-fold less than that of NO_2^- reduction to NH_3^+. The nitrate reductase, NarG, generates NO in the presence of nitrite and in the absence of nitrate as a side-product from nitrite reduction (Calmels *et al.,* 1988; Ralt *et al.*, 1988; Metheringham and Cole 1997; Gilberthorpe and Poole, 2008; Vine *et al*., 2011). However, some NO can be also formed from nitrite even in mutants that lack NarGHJI. It has therefore been suggested that NO is generated inside the cell from nitrite by one or both of the nitrite reductases. Indeed, Weiss (2006) reported that NirB might be responsible for the majority of NO production in the *E. coli* cytoplasm during anaerobic nitrate or nitrite metabolism. However, Corker and Poole (2003) concluded that NO is mainly produced by NrfA as a side-product during reduction of nitrite to ammonia. In contrast, Vine *et al.* (2011) found that both NirB and NrfA have a vital role in preventing the build-up of NO

in the cytoplasm rather than its accumulation. Before this study, it was unknown whether the di-iron protein YtfE is another source of intracellular NO. Proteins homologues to *E. coli* YtfE such as DnrN and ScdA are widely distributed in nature, including *Staphylococci* and *Neisseria* species (Justino *et al.,* 2007; Overton *et al*., 2008). It has been suggested that YtfE plays a critical role in survival of *Haemophilus influenzae* in macrophages (Harrington *et al*., 2009). Several studies have concluded that YtfE and its homologues play a general role in the repair of damaged iron-sulfur proteins following either oxidative or nitrosative stress damage (Justino *et al*., 2006; Justino *et al.,* 2007; Overton *et al*., 2008; Justino *et al*; Justino *et al*., 2009; Nobre *et al.,* 2014). Although the mechanism of damage repair remains undetermined, work by Nobre e*t al*. (2014) has shown that Fe^{++} from YtfE can be transferred to iron-deficient iron-sulfur centres following oxidative or nitrosative damage.

The rate of iron transfer is extremely slow, and this suggested role of YtfE does not explain how nitrosylated or oxidatively damaged iron atoms in proteins are released before the proposed repair function can be completed. Alternative possible roles for YtfE have not been investigated, namely that it is an enzyme that catalyses the release of Fe-NO, or even NO itself, from iron-sulfur proteins that have been nitrosylated by NO generated by nitrite reduction.

In view of these more recent observations, the aims of this chapter are therefore first to re-exam the phenotypes of mutants defective in *ytfE* during nitrosative stress and also determine other sources of nitric oxide. Previous studies were completed with a strain that carried both a *ytfE* mutation and an additional deletion of 126 genes at a secondary locus (Vine *et al*., 2010). This secondary deletion included many genes that are essential for anaerobic respiratory functions, for example, the *moa* and *mod* operons required for

molybdopterin synthesis, and hence for formate dehydrogenase, nitrate reductase and TMAO reductase synthesis. Discovery of this secondary deletion readily explained the complex phenotypes and growth defects of the original *ytfE* mutant (Vine *et al*., 2010). Critically, however, the deletion also included the *hcp-hcr* operon, and we recently reported that Hcp plays an essential role in a high affinity but low capacity nitric oxide reduction system that, in the absence of other known NO reductases, is essential for growth under conditions of nitrosative stress in the physiologically relevant nM range (Wang *et al*., 2016; in submission). Therefore, for many of the experiments presented in this chapter, the effect of *ytfE* mutation will be compared with the effect of *hcp* mutations to determine which mutation is more important.

4.2 Effect of nitric oxide on growth of strains defective in Hcp or YtfE

The relative effects of *ytfE* and *hcp-hcr* mutations on sensitivity to nitrosative stress were investigated. For these experiments, the parental strain, RK4353, the *ΔytfE* strain JCB5211, the *Δhcp* strain, JCB5000 and the *ytfE hcp* double mutant, JCB5228 were grown anaerobically in minimal salts medium at 37ºC in the presence and absence of a range of NO concentrations (5 µM, 10 µM and 20 µM). The growth of the four strains was monitored for 7 hours and then compared with that of unsupplemented controls. All four strains grew well in the presence and absence of 5 µM NO (Figure 4.1, A, B, C, D). Growth of all four strains was partially inhibited by 10 µM of NO (Figure 4.1 A, B, C, D). In contrast, repeated additions of 20 µM NO caused complete inhibition of growth (Figure 4.1 A, B, C, D). These results indicated that YtfE is not essential for protecting *E. coli* exposed to exogenously added nitric oxide even in the mutant that is deficient in Hcp.

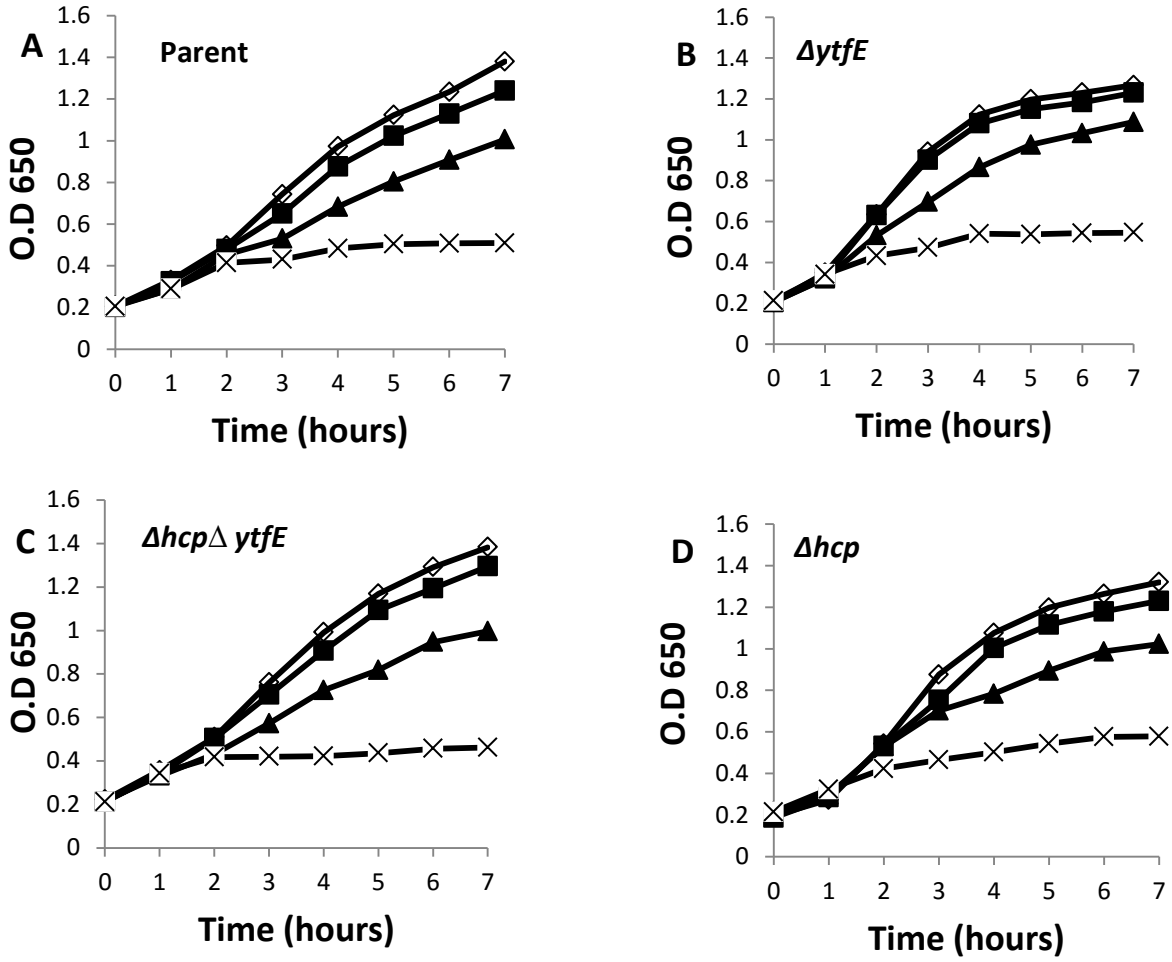


Figure 4.1: A, B, C, D: Growth phenotype of strains defective in the di-iron protein, YtfE, and the hybrid cluster protein, Hcp, in medium supplemented or unsupplemented with different concentrations of NO

The parental strain, RK4353, the *ΔytfE* strain, JCB5201, the *Δhcp* strain, JCB5000 and the *ΔytfE::cat Δhcp::cat* strain, JCB5228 were grown anaerobically in the minimal salts medium at 37ºC in the presence of 5 µM of NO (squares), 10 µM of NO (triangles) and 20 µM of NO (crosses symbols) or in the absence of NO (diamonds symbols). The optical density for each culture was measured every hour for 7 hours. This graph shows results from a typical experiment from biological replicates completed on different days and with different batches of medium.

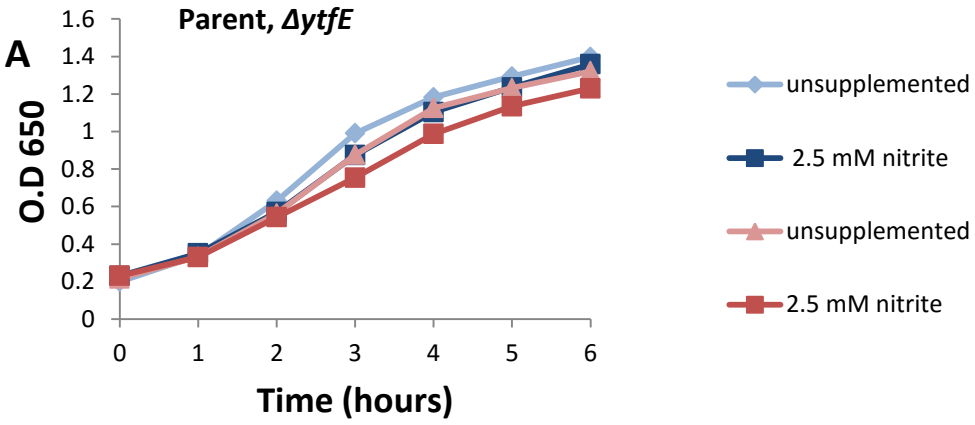
In order to investigate whether nitrite also affects the growth of the same set of strains, the same four strains were grown anaerobically at 37ºC in the presence and absence of 2.5 mM nitrite. The growth of the mutants was not suppressed by nitrite compared to that of parental strain, RK4353, in which both *hcp* and *ytfE* are functional (Figure 4.2, A, B, C). These preliminary experiments indicated that neither Hcp nor YtfE is essential for anaerobic growth in the presence of NO or nitrite when other protective mechanisms against nitrosative stress such as NorVW, NrfAB and Hmp were functional.

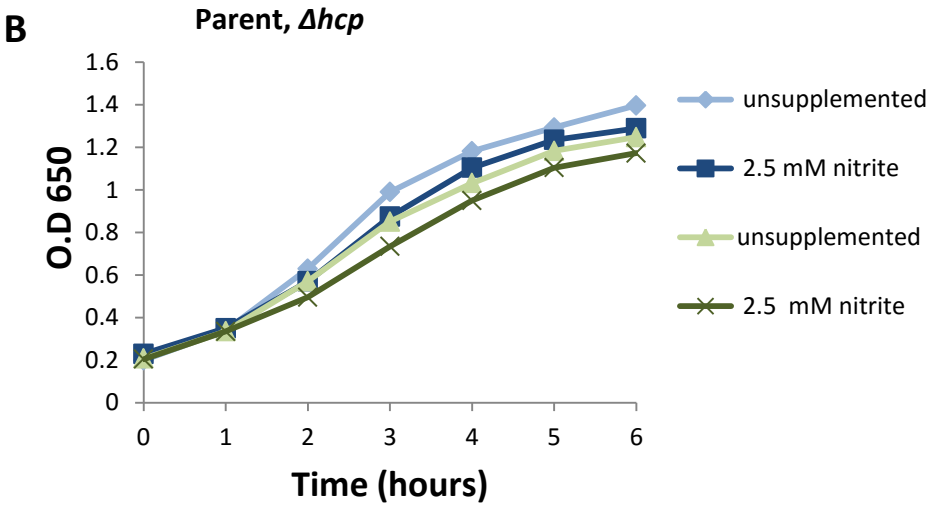
4.3 The effect of a *ytfE* mutation on the anaerobic growth of a strains lacking all known nitrite and NO reductase activities

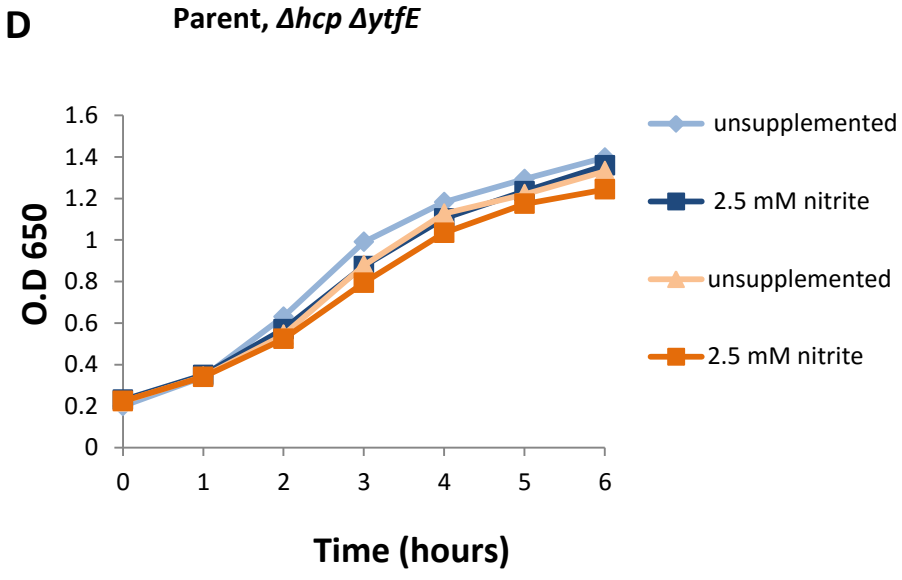
It seemed likely that the lack of a strong phenotype of mutants lacking YtfE or Hcp in the above experiments was due to the ability of multiple other proteins to remove cytoplasmic NO by reduction to ammonia or nitrous oxide. To generate a set of four strains that are more sensitive to oxidative or nitrosative stress, Dr. Vine transferred *hcp* and *ytfE* mutations into the mutant strain JCB5210 that lacks both of the *E. coli* nitrite reductases, *nirBD* and *nrfAB* genes, as well as the *hmp* and *norVW* genes (Table 3.1). The set of four strains were then grown anaerobically in the presence and absence of nitrite. Both of the Hcp^- strains were extremely sensitive to nitrosative stress but the corresponding $YtfE^-$ strain was resistant (Vine, 2012). In order to further investigate the role of YtfE in protection against nitrosative stress during anaerobic growth, first the effect of NO was determined by growing the four strains anaerobically in the presence or absence of various concentrations of NOSW. To determine the concentration of NO that will inhibit but not prevent the growth of the *hcp ytfE* strain, JCB5260, this strain was grown anaerobically in

Figure 4.2: A, B, C: The effect of *hcp* and *ytfE* mutation on growth of *E. coli* in the presence of nitrite.

The parent strain, RK4353 (blue), the *ΔytfE* strain, JCB5201 (red), the *Δhcp* strain, JCB5000 (green) and the Δ*ytfE::cat Δhcp::cat* strain, JCB5228 (orange) were grown anaerobically in MS at 37ºC in the presence of 2.5 mM nitrite (the dark coloured lines) or in the absence of nitrite (the pale coloured lines). This graph shows data from a typical biological replicate completed on different days and with different batches of medium. Other details are as in Figure 4.1.


A
Parent, ΔytfE
O.D 650
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6
Time (hours)
unsupplemented
2.5 mM nitrite
unsupplemented
2.5 mM nitrite


B
Parent, Δhcp
O.D 650
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6
Time (hours)
unsupplemented
2.5 mM nitrite
unsupplemented
2.5 mM nitrite


D
Parent, Δhcp ΔytfE
O.D 650
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6
Time (hours)
unsupplemented
2.5 mM nitrite
unsupplemented
2.5 mM nitrite

1 L of minimal salts medium at 37ºC either in the presence or absence of different concentrations of NO. The mutant strain, JCB5260, grew well in the presence and absence of 1 µM NO. However, 2 µM and 4 µM NO strongly inhibited the growth of this strain (Figure 4.3). It was therefore decided to use 1.5 µM of NO for future experiments.

Addition of 1.5 µM NO completely inhibited the growth of the $Hcp^{-}YtfE^{+}$ strain, JCB5250 (Figure 4.4, B) while mutation in both *hcp* and *ytfE* resulted in a strain that was able to grow albeit slowly (Figure 4.4, D). However, growth of the $Hcp^{+}YtfE^{+}$ and $Hcp^{+}YtfE^{-}$ strains was unaffected (Figure 4.4 A, C). These results suggest that YtfE, which was previously proposed to be a major factor in providing protection against nitrosative stress, had no detectable effect on the survival of the four strains exposed to nitrosative stress. However, YtfE is partially responsible for the extreme toxicity of NO to a strain that lacks Hcp in addition to other known mechanisms of NO reduction. In contrast, Hcp is the key player in rendering this protection.

4.4 Effect of a *ytfE* mutation on expression from the *hcp* promoter in strains defective in all nitrite and nitric oxide reductases

If YtfE generates NO, nitrite or NO will induce transcription from the *hcp::lacZ* promoter fusion plasmid, so the resulting β-galactosidase activity will be higher in a $YtfE^{+}$ strain than that in $YtfE^{-}$ strain. To test this hypothesis, the set of four strains (genotypes $hcp^{+}ytfE^{+}$, *hcp* $ytfE^{+}$, hcp^{+} *ytfE*, and *hcp ytfE*) that were defective in all known NO and nitrite reductases were transformed with the *hcp* promoter-*lacZ* fusion plasmid, pNF383. Purified transformants were grown under anaerobic conditions at 37ºC with either 0.2 mM nitrite or

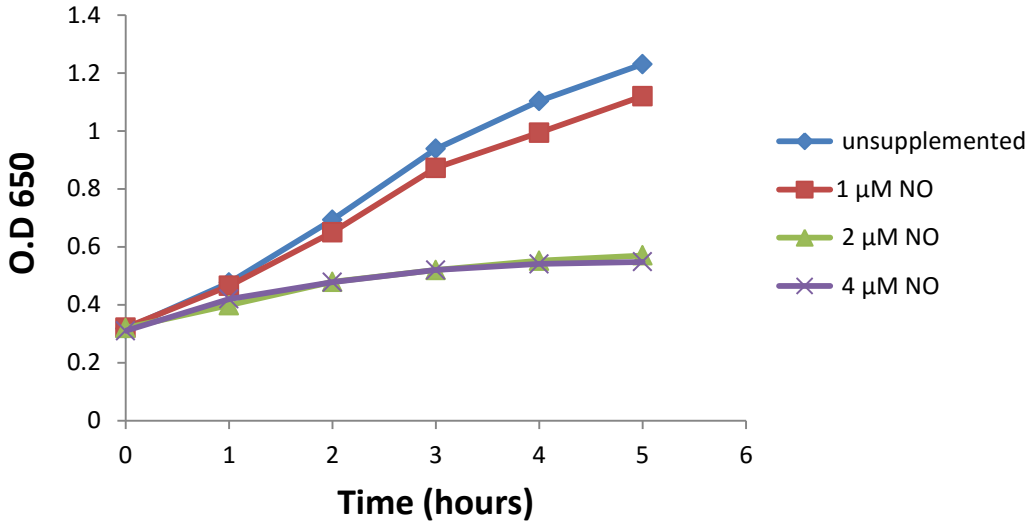


Figure 4.3: Preliminary experiment for determination of NO concentration used for imposed nitrosative stress for further experiments

The *hcp ytfE* strain, JCB5260, was grown anaerobically at 37ºC in 1 L of MS until the optical density at 650 nm had reached 0.25. Cultures were then supplemented with 1 µM NO (red line), 2 µM NO (Green line) or 4 µM NO (purple line). The fourth culture (blue line) was left as an unsupplemented control. This graph shows data from a typical biological replicate completed on different days and with different batches of medium. Other details are as in Figure 4.1.

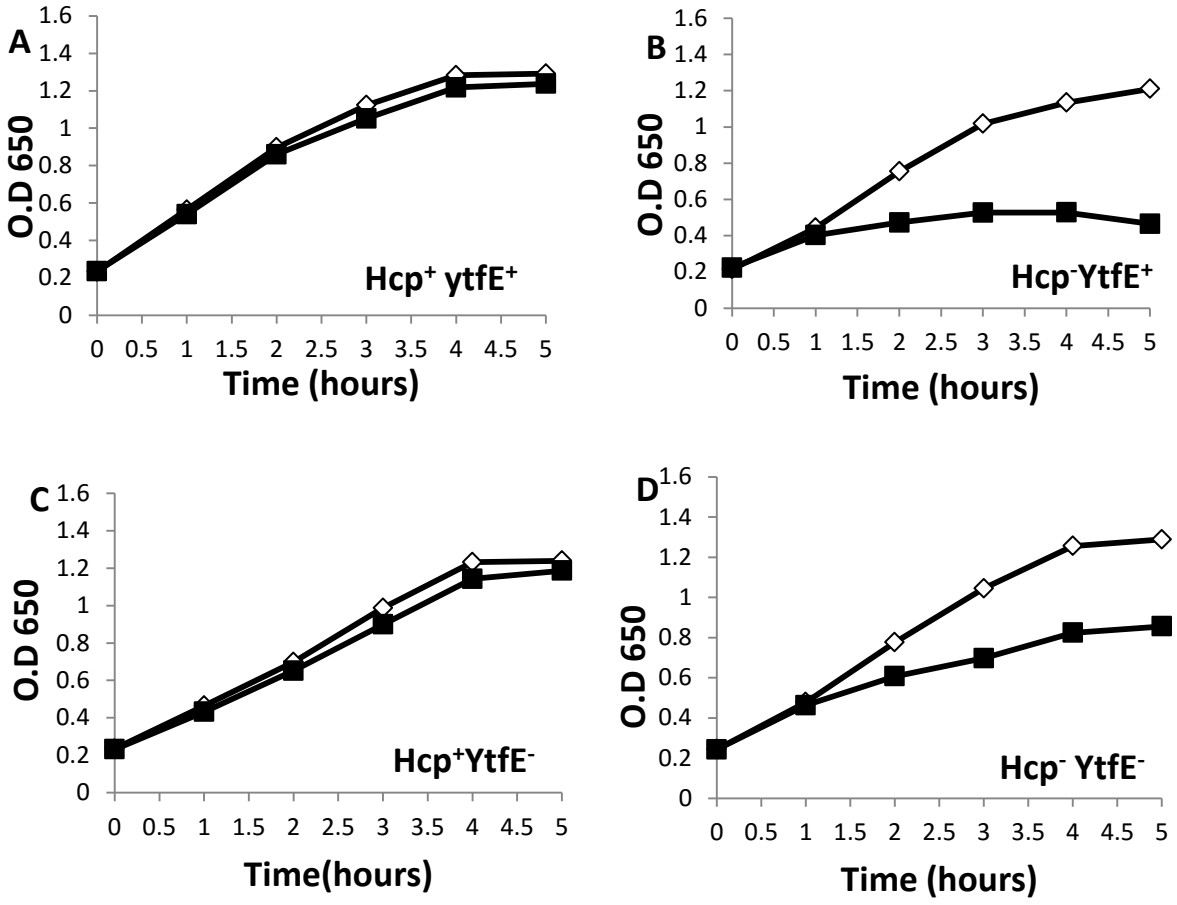


Figure 4.4: Effect of NO–saturated water on anaerobic growth of strains containing all combinations of *hcp* and *ytfE* mutations

An isogenic set of four strains with all four combinations of the *ytfE* and *hcp* genes were grown anaerobically in minimal salts medium in the presence of 1.5 µM of NOSW, which was added repeatedly every 30 minutes (filled symbols) or in the absence of NOSW (opened symbols). The optical density was monitored at hourly intervals for 5 hours. This graph represents a typical experiment of biological replicates completed on different days and with different batches of medium.

exogenous NO to a final concentration 0.1 μM, which was added repeatedly every 30 minutes. Samples (1 ml) were lysed just before addition of nitrite and nitric oxide, and then 2 hours after nitrite and NO addition to be assayed for β-galactosidase activity, as previously described. After 2 hours, the activity of the *hcp* promoter in the Hcp^- $YtfE^+$ and Hcp^- $YtfE^-$ strains was higher than that in the Hcp^+ $YtfE^+$ and Hcp^+ $YtfE^-$ strains in the presence of nitrite or NO (Figure 4.5, A, B). These observations were consistent with inactivation of NsrR by NO generated from nitrite or supplied as NOSW. These results confirmed that Hcp removes NO in Hcp^+ $YtfE^+$ and Hcp^+ $YtfE^-$ strains, while YtfE generates NO in the Hcp^- $YtfE^+$ strain.

4.5 Effect of YtfE on *in vivo* aconitase activity during anaerobic growth

Aconitase is one of the labile [4Fe-4S] dehyratases that has a vital role in the reactions of energy generation within the tricarboxylic acid cycle (TCA cycle) that is fundamental in controlling cellular metabolism. In the TCA cycle, this enzyme isomerizes citrate to isocitrate via cis-aconitate (Krebs and Holzach, 1952). The iron-sulfur centres of *E. coli* aconitase are very sensitive to NO (Gardner *et al*., 1997). We also recently reported that aconitase activity is much lower or almost absent from cultures of the Hcp^- $YtfE^+$ strain, JCB5250, during growth in the presence of nitrate or NO (Wang *et al*., 2016). If this inactivation is due to NO released by YtfE from nitrosylated iron-sulfur proteins, the aconitase activity of the strains that lack YtfE should be higher during growth in the presence of nitrosative stress than the isogenic $ytfE^+$ strain. However, Hcp should be essential to remove cytoplasmic NO as rapidly as it is released To test this prediction, the bacterial cultures of the four isogenic strains used for the growth experiment were harvested, washed with phosphate buffer and broken by sonication. After centrifugation at high speed, the soluble supernatant was assayed for aconitase activity.

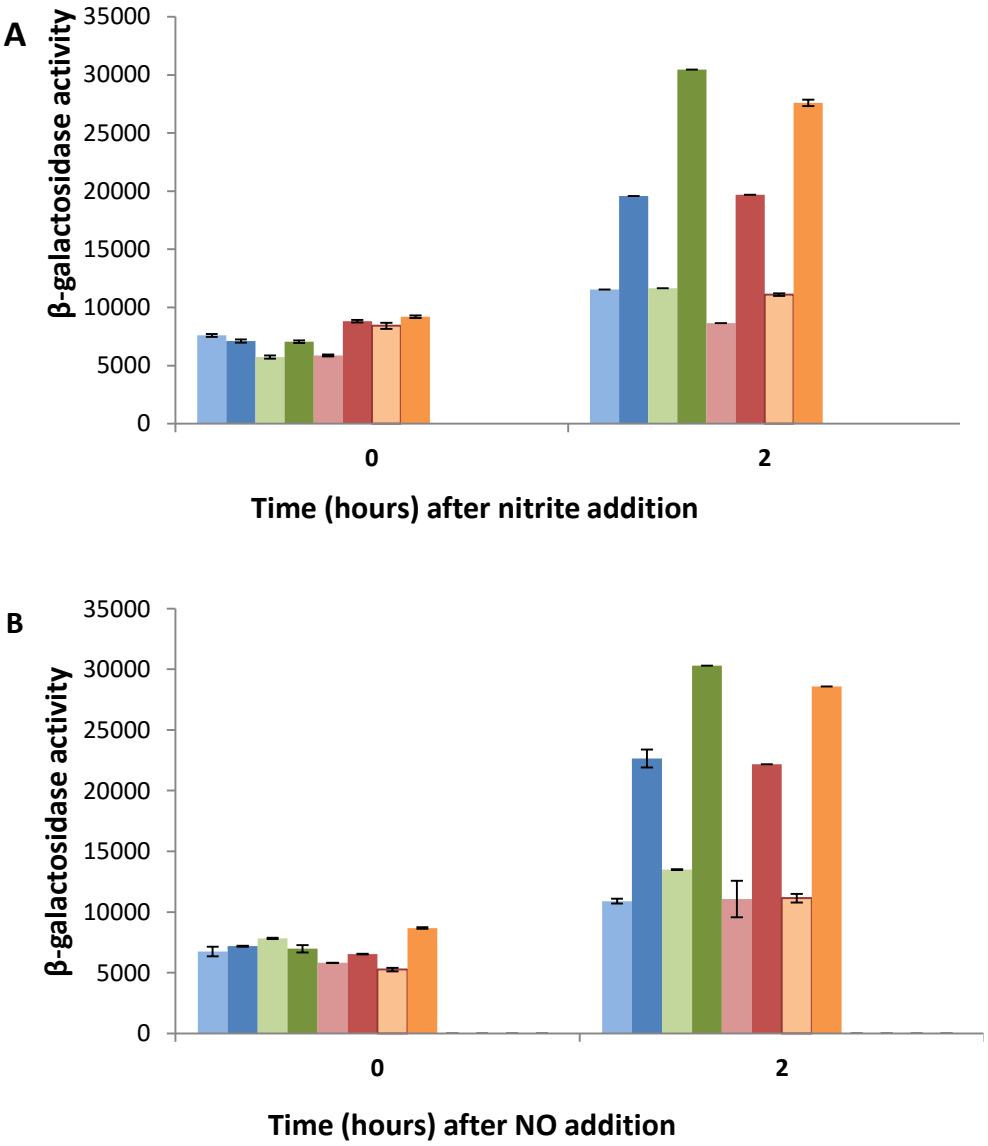


Figure 4.5: The effect of nitrite and NO on transcription of from the *hcp* promoter in strains defective in all nitrite and NO reductases

The set of four strains *hcp*$^{+}$*ytfE*$^{+}$ (blue), *hcp ytfE*$^{+}$ (green) *hcp*$^{+}$ *ytfE* (red) and *hcp ytfE* (orange), all of which are also deleted for *nirBD*, *nrfAB*, *norV* and *hmp* genes were transformed with *hcp::lacZ* promoter fusion plasmid, pNF383 and then grown anaerobically in MS either unsupplemented (pale bars), or supplemented (dark bars) with 0.2 mM sodium nitrite or 0.1 µM NO. Cells were lysed and assayed for β-galactosidase activity. Other details are as in Figure 3.1.

Aconitase activity was almost completely inhibited after anaerobic growth of the two Hcp-deficient strains, JCB5250 and JCB5260, in the presence of 1.5 μM NO (Figure 4.6, A, B). This effect was largely dependent upon the absence of Hcp rather than YtfE. However, aconitase activity was readily detectable in both of the *hcp*$^{+}$ strains, JCB5210 and JCB5257 (Figure 4.6, A, B). These results suggested that YtfE does not fulfil a major role in protection against nitrosative stress as previously reported but instead it releases free NO from proteins damaged by nitrosylation. In addition to this, Hcp is a critical factor in reducing the toxic product that is released by YtfE.

4.6 The effect of YtfE on *in vivo* fumarase activity during anaerobic growth

Fumarase B is another iron-sulfur enzyme that plays an important role in intermediary metabolism during anaerobic growth. It is also inactivated by NO (Girsch and de Vries, 1997; Justino *et al.,* 2007). This enzyme catalyses the reversible hydration of fumarate to malate in the TCA cycle. In *E. coli,* there are three fumarase isozymes, FumA, FumB and FumC. FumB is class one fumarase that acts efficiently anaerobically and provides fumarate as an electron acceptor. The iron-sulfur clusters of this enzyme are very susceptible to nitrosation by nitric oxide (Woods *et al*., 1988). In order to check whether the effect of NO is specific for one iron-sulfur protein or a more general phenomenon, fumarase activity, was also assayed in the extracts prepared from the same cultures used for aconitase assays.

There was a similar effect of NO addition on fumarase activity. However, the inhibition addition of fumarase activity by NO was less severe than for aconitase activity. Significantly, however, fumarase activity was extremely low in both of the *Δhcp* strains JCB5250

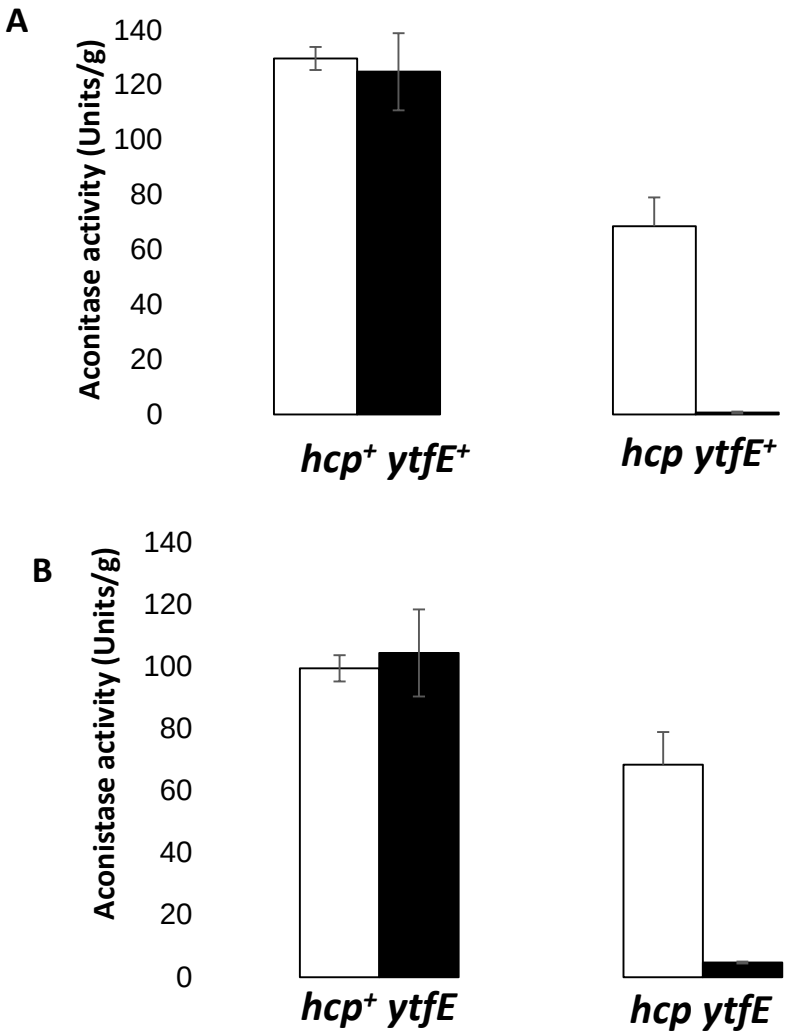


Figure 4.6: Inactivation of aconitase by NO in strains that lack all nitrite and NO reductase

The set of four strains (genotypes *hcp*$^{+}$*ytfE*$^{+}$; *hcp ytfE*$^{+}$; *hcp*$^{+}$*ytfE*; and *hcp ytfE* deleted for *nirBD*, *nrfAB*, *norV* and *hmp*) were grown anaerobically in cultures either unsupplemented (open bars), or supplemented (filled bars) with 1.5 µM NO added as NO-saturated water every 30 min. Note that the aconitase activity of the two strains Hcp^{-}YtfE^{+}, JCB5257, and Hcp^{-} YtfE^{-}, JCB5260 was extremely low after growth in the presence of 1.5 µM NO. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean+/- standard deviation).

(p=0.0001) and JCB5260 (p=0.003) after growth in cultures pulsed with NO (Figure 4.7, A, B). This further indicates that YtfE generates a toxic product, NO, that is reduced by Hcp.

4.7 Effect of a *ytfE* mutation on transcription at an NsrR-repressed promoter in different strains

The nitric oxide sensor-regulator, NsrR, plays a major role in this stress response. Microarray analysis showed that NsrR in *E. coli* represses about 20 promoters, including *hmpA*, *ytfE*, *ygbA,* and the *hcp-hcr* operon (Bodenmiller and Spiro, 2006; Filenko *et al*., 2007). NsrR represses transcription at the *hcp* promoter, but repression is relieved by cytoplasmic NO generated as a side product of nitrite reduction (Filenko *et al.*, 2007; Vine *et al*., 2011). Therefore, the effects of a *ytfE* mutation on the activation of the *hcp* promoter in the wild type, RK4353, and RK4353 *ytfE*::*cat* strains were compared. Both strains were transformed with plasmid pNF383 in which the *hcp* promoter was fused to *lacZ* in the promoter-probe vector, pRW50, and were grown anaerobically until the optical density at 650 nm had reached 0.2. One culture was then supplemented with 2.5 mM nitrite while the other was left as an unsupplemented control. The samples (1 ml) were then lysed and assayed for β-galactosidase activity every hour for 2 hours. The β-galactosidase activity due to transcription of *lacZ* from the *hcp* promoter during growth in the presence of nitrite was significantly higher in the parental strain than in the *ytfE* mutant (p= 0.0062) (Figure 4.8). This was further evidence that the YtfE protein causes accumulation of intracellular nitric oxide during the repair of iron–sulphur centres of proteins damaged by nitrosylation.

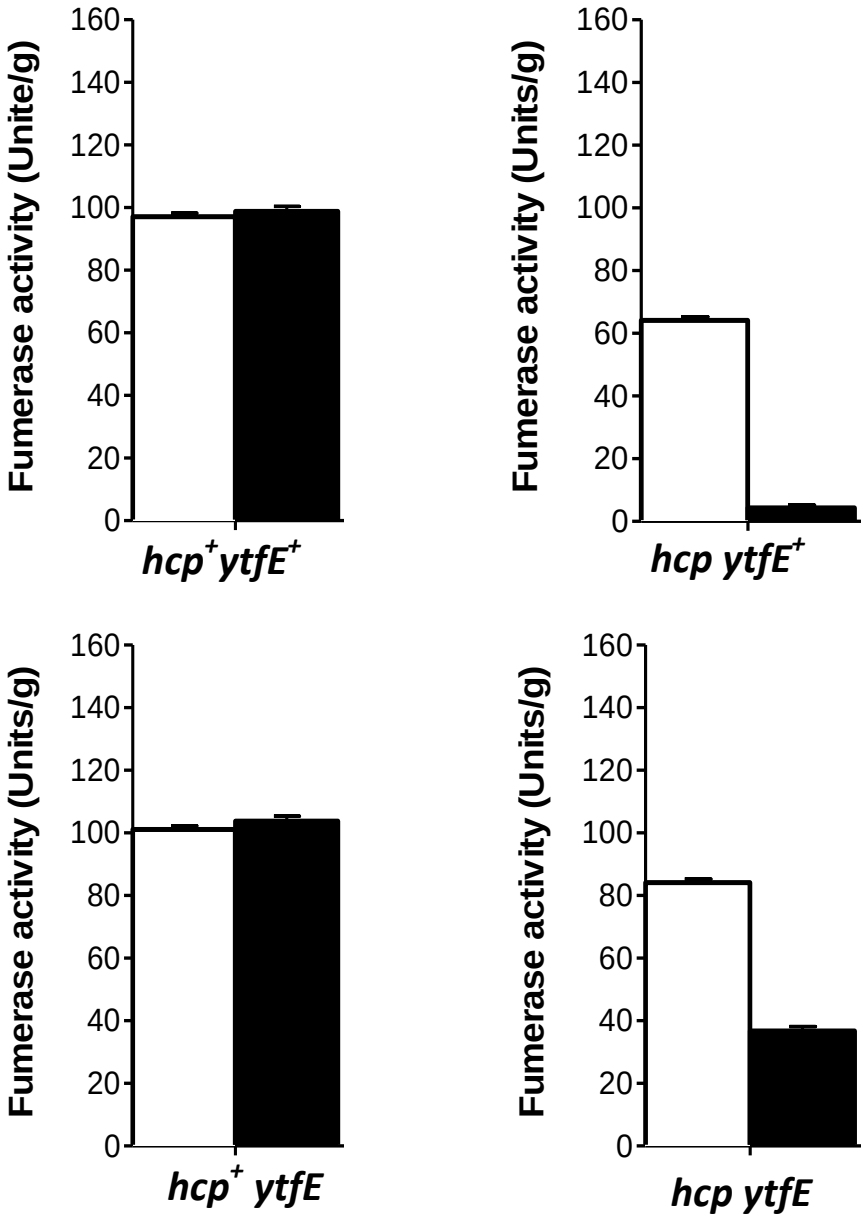


Figure 4.7: Inactivation of fumarase in strains that lack all nitrite and NO reductases

The set of four strains (genotypes *hcp*$^{+}$*ytfE*$^{+}$; *hcp ytfE*$^{+}$; *hcp*$^{+}$*ytfE*; and *hcp ytfE* deleted for *nirBD*, *nrfAB*, *norV* and *hmp*) were grown under anaerobic conditions at 37ºC in minimal salts medium in the presence of 1.5 µM NO (filled bars), or in the absence of NO (open bars) which was added repeatedly every 30 min. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation). Other details are as in Figure 4. 6.

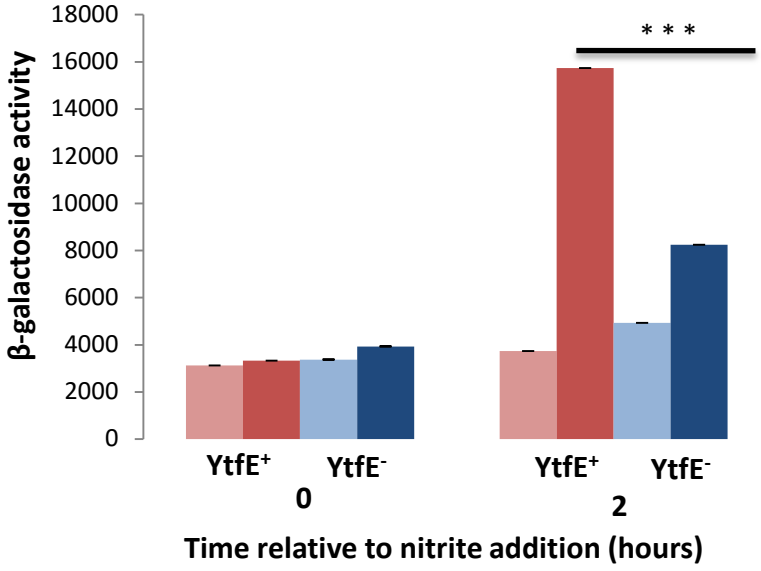


Figure 4.8: Nitric oxide generation as reported by *hcp* promoter activity in bacteria defective in the YtfE protein

The parental strain, RK4353 (red), and the RK4353 *ΔytfE*::cat strain (blue) were transformed with pNF383. The strains were grown in duplicate under anaerobic conditions in the presence of 2.5 mM nitrite, which was added when the optical density had reached 0.2 at 650 nm (dark red, and dark blue bars). Control cultures were grown in the absence of nitrite (pale red, and pale blue bars). Samples (1 ml) were then lysed and assayed for β-galactosidase activity. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation). *,**,*** represents significance ($P<0.05$), ($P<0.01$) and ($P<0.005$) respectively .

Vine (2012) found that some NO was still accumulating in the double mutant, JCB4022 that was deficient in *narG* and *napAB*. Therefore, if YtfE is responsible for the production of the residual NO during the repair of damaged iron-sulfur centres in this strain, the activity of β-galactosidase would be lower in a triple mutant *napAB narG ytfE* than in the double mutant strain, JCB4022. To test this hypothesis, a triple mutant that is defective in the *napAB*, *narG*, and *ytfE* was required. Bacteriophage P1 transduction was used to transfer the *ytfE* mutation from the RK4353 Δ*ytfE::cat* mutant into the RK4353 *narG napAB* strain, JCB4022. Colony PCR was used to confirm that the transductants contained the correct chloramphenicol resistance cassette (*cat*) in place of the *ytfE* gene. After transduction with P1, the wild type, RK4353, *narG napAB* JCB4022 and its derivative *napAB narG ytfE* strain, JCB5300, were transformed with pNF383 and then grown anaerobically in the presence and absence of 2.5 mM of nitrite. The β-galactosidase activity was assayed as previously described.

As expected, the activation of the *hcp* promoter in the strain JCB5300 was lower than that in strain JCB4022; this decrease was statistically significant (p=0.0006) (Figure 4.9). In contrast, the induction of the *hcp* promoter in the parent strain was higher than double mutant (p=0.0060) and triple mutant (p=0.003) (Figure 4.9). Once again, this suggested that YtfE is another source of cytoplasmic NO. The data also confirmed that less NO is generated in strains that lack the nitrate reductase, NarG, than in the parent strain.

It was also interesting to determine the effect of all combinations of *ytfE* and *hcp* mutations on the expression of the *hcp* promoter. To this end, the parental strain, RK4353, the *hcp* strain, JCB5000, the RK4353 *ΔytfE::cat* strain, JCB5201, and the *ytfE hcp* double mutant, JCB5228, were transformed with pNF383. The set of four strains that contained all combinations of the presence and absence of Hcp and YtfE was then grown anaerobically in

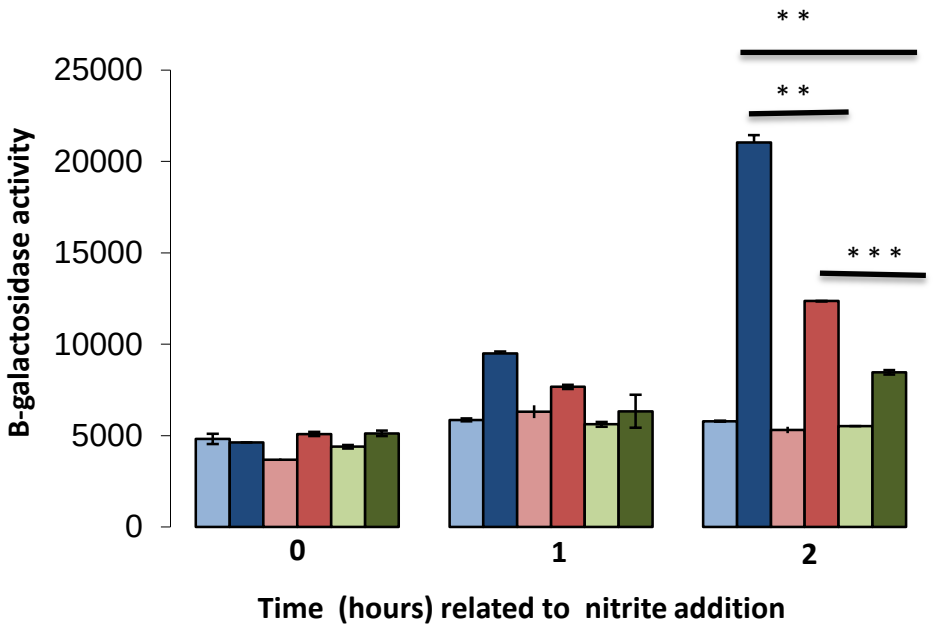


Figure 4.9: Effect of YtfE and NarG on accumulation of NO in the cytoplasm

The wild type, RK4353 (blue), the *narG napA* strain, JCB4022 (red) and *narG napA ytfE*, JCB5300 (green), were transformed with pNF383. Transformants were grown anaerobically in MS medium in the presence of 2.5 mM nitrite (dark blue, dark red and dark green bars) and in absence of nitrite (pale blue, pale red and pale green bars). Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation). Other details are as in the Figure 4.8.). *,**,*** represents significance ($P<0.05$), ($P<0.01$) and ($P<0.005$) respectively .

the minimal salts medium in the presence or absence of 2.5 mM sodium nitrite (Figure 4.10). After 2 hours, eight cultures were then lysed for determination of β-galactosidase activities. As previously reported, nitrite induced β-galactosidase synthesis relative to the unsupplemented control culture, but higher induction was observed in the Hcp-deficient strain, JCB5000, than in the parent strain; this increase was statistically significant (p=0.006) (Figure 4.10). In contrast, far less nitrite induction of transcription at the *hcp* promoter was observed in strain JCB5211, which lacks YtfE (p=0.0003) compared to the parent strain. Induction was restored in the *hcp ytfE* double mutant, strain JCB5228, because Hcp was no longer available to remove cytoplasmic NO generated by various mechanisms (Figure 4.10).

4.8 The role of YtfE in the generation of NO in strains defective in all nitrate, nitrite and NO reductases and HCP

Previous studies and data presented in (Figure 4.9) have established that a major source of cytoplasmic NO is due to a side reaction catalysed by the cytoplasmic nitrate reductase, NarG, in which nitrite is reduced to NO. Some iron-sulphur proteins immediately trap this NO, resulting in the inactivation of key metabolic functions and growth inhibition (Seth *et al*., 2012; Vine *et al*., 2011).

To investigate whether YtfE is also able to produce NO in the strain defective in all previously characterised nitrate, nitrite and NO reductases and Hcp, the *ytfE*$^{-}$ strain, JCB5280, and the *ytfE*$^{+}$ strain, JCB5270, were grown anaerobically in the presence and absence 1 mM sodium nitrite. Both strains grew well in the absence of nitrite (Figure 4.11, A, B). and the *ytfE* mutant, JCB5280, grew very well even in the presence of 1 mM sodium nitrite Figure 4.11, A). However, a very interesting phenotype appeared when YtfE was present, as growth

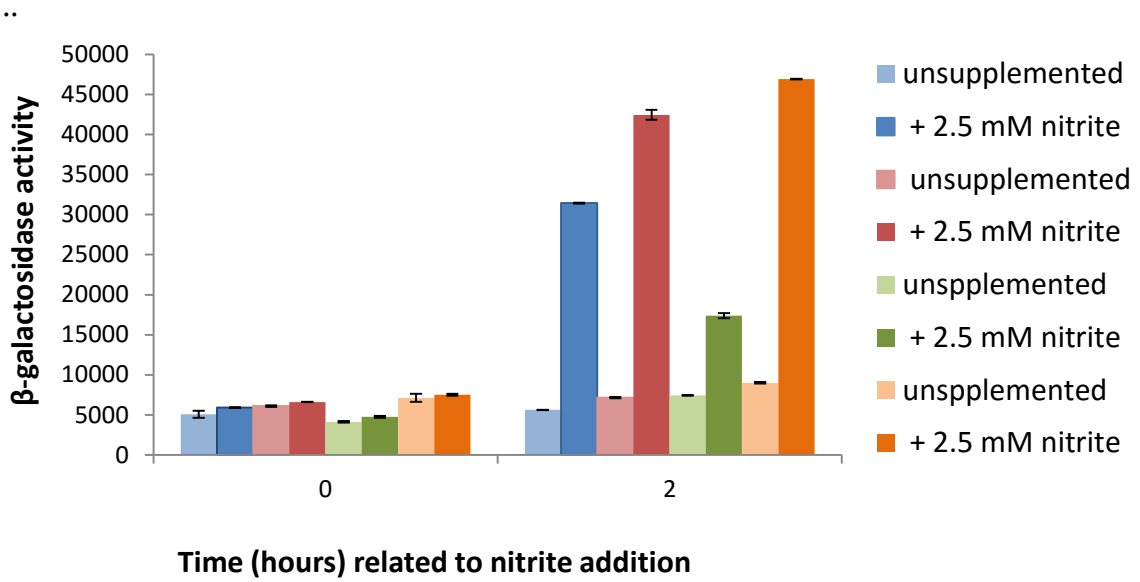


Figure 4.10: Detection of NO accumulated in *hcp* and *ytfE* mutant strains

The parental strain, RK4353 (blue), the *hcp* mutant JCB5000 (red), the *ytfE* mutant JCB5211 (green), and the *ytfE hcp* double mutant, JCB5228 (orange) were transformed with pNF383. The cultures were grown anaerobically in MS medium that was unsupplemented (pale blue, pale red, pale green and pale orange bars), or supplemented with 2.5 mM sodium nitrite (dark blue, dark red, dark green and dark orange bars). The β-galactosidase activity for each culture was assayed as previously described. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation). Other details are as in Figure .3.1.

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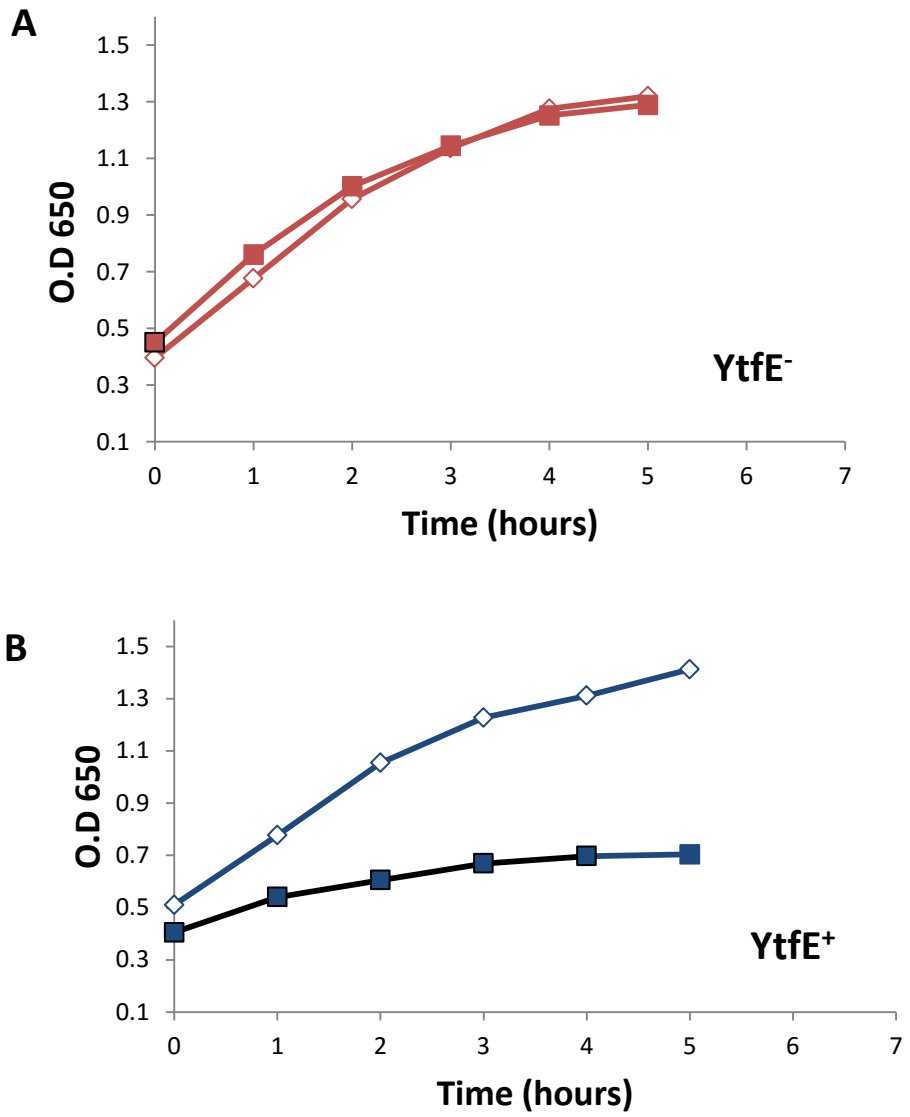

Figure 4.11: The effect of *a ytfE* mutation on anaerobic growth of strains that defective in all nitrate, nitrite and NO reductases and Hcp in the presence of 1 mM nitrite

The *narZ narG nrfA hmp norV nirB hcp ytfE*, JCB5280 (blue), and the *narZ narG nrfA hmp norV nirB hcp* strain JCB5270 (red), were grown anaerobically at 37º C without shaking in the presence of 1 mM nitrite (filled symbols) or absence of 1 mM nitrite (open symbols). The optical density for each culture was measured every hour for 5 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

of the *ytfE*$^{+}$ strain was almost totally inhibited by 1 mM nitrite (Figure 4.11, B). This was the strongest phenotype that had yet been reported for the *ytfE* mutant. This again indicates that YtfE is another source of nitric oxide, which causes deficiency in growth of the *ytfE*$^{+}$ strain, JCB5270.

It was also important to determine whether NO in addition to nitrite also inhibited the growth of the *ytfE*$^{+}$ strain. Both strains were grown anaerobically as previously described in minimal salts medium in the presence or absence of 1 mM of nitrite and different concentrations of NO (1 and 5 µM), which was added repeatedly every 30 minutes. Both strains grew very well in the absence of NO and nitrite and in the presence of 1 µM of NO (Figure 4.12). In contrast, the growth of both strains was inhibited in the presence of 5 µM of NO (Figure 4.12). In addition, the growth of strain JCB5270 was inhibited when the medium was supplemented with nitrite, while no effect was detectable on strain JCB5280 as shown in the previous data (Figure 4.11, A, B). Again, these results indicated that poor growth of strain JCB5270 in the presence of sodium nitrite might be due to the toxicity of NO that was generated by YtfE during the repair of damaged iron-sulphur centres as in previous results. Additionally, the sensitivity of both strains to 5 µM of exogenous NO was due to the absence of Hcp and NO reductases that protect against the toxicity of NO.

4.9 The effect of *ytfE* mutation on transcription from the *hcp* promoter in strains defective in all nitrate, nitrite and nitric oxide reductases and Hcp

If the toxic product that inhibited the growth of *ytfE*$^{+}$ strain JCB5270 is NO (Figure 4.11), the expression of *hcp::lacZ* should be more than in the *ytfE* strain, JCB5280.

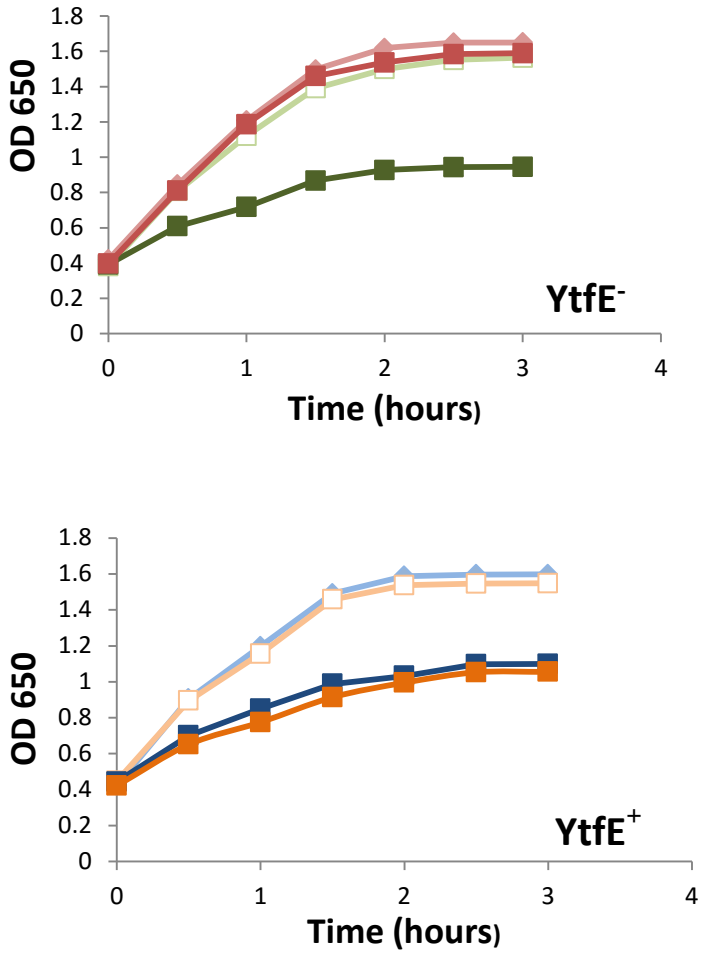

Figure 4.12: The effect of *ytfE* mutation on anaerobic growth of strains defective in all nitrate nitrite and nitric oxide reductases and Hcp in the presence of nitrite and NO

RK4353 *narZ narG nrfA hmp norV nirB hcp ytfE*, JCB5280 (red), and the *narZ narG nrfA hmp norV nirB hcp* strain JCB5270 (blue), were grown in MS anaerobically at 37ºC without shaking either in the absence of 1 mM of nitrite (pale red and pale blue symbols) or in the presence of nitrite (dark red and dark blue symbols) or in the presence of different concentrations of NOSW 1 µM (the pale open green and pale open orange symbols) and in the presence of 5 µM (the dark closed green and dark closed orange symbols). The optical density for each culture was measured every hour for 3 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

To test this hypothesis, both strains were transformed with the pNF383 plasmid. Bacteria were grown in minimal salts medium under anoxic conditions in the presence or absence of 0.5 mM sodium nitrite. The β-galactosidase activities were determined and the optical density was monitored for 4 hours. The activity of the *hcp* promoter in the strain defective in YtfE was lower in the presence of nitrite than when YtfE was functional, this decrease was statistically significant (p=0.0082) (Figure 4.13). These data suggest that expression from the *hcp* promoter in the *ytfE*$^+$ strain, JCB5270 is induced by NO that is liberated from the repair of damaged iron-sulfur clusters of some proteins by the di-iron protein, YtfE. Nevertheless, nitrite induction in the *ytfE*$^+$ strain indicated that some NO was still being accumulated in the cytoplasm, despite the absence of all nitrate and nitrite reductases.

4.10 Restoration of sensitivity to nitrosative stress by complementation with a *ytfE*$^+$ plasmid

For further confirmation that the growth inhibition observed in the *ytfE*$^+$ strain was due to NO generated by the YtfE protein, complementation of the *ytfE* defect of strain JCB5280 with a multi-copy expression plasmid expressing only *ytfE*$^+$ should restore growth inhibition by NO generated from nitrite. To test this prediction, the *ytfE*$^+$ plasmid (pACYC184) was transformed into the *ytfE*$^+$ strain JCB5270 and its *ytfE* derivative strain, JCB5280, both after curing them with pCP20 to remove their antibiotic resistance cassettes. Transformants and untransformed control strains were grown anaerobically in minimal salts medium under anaerobic conditions in the presence or absence of 1 mM nitrite and their growth was compared with that of the untransformed host strains. Unexpectedly, growth of the transformants was highly inhibited both in the presence and absence of nitrite (Figure 4.14, A, B).

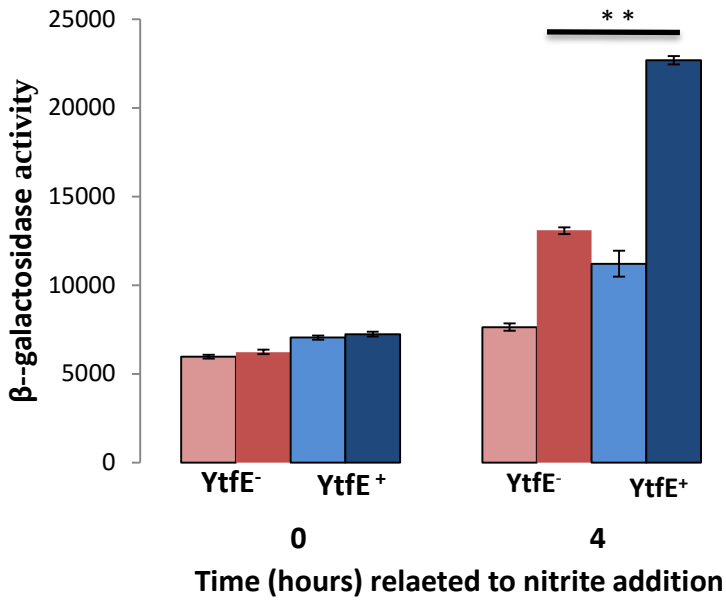

Figure 4.13: Nitric oxide generation as reported at *hcp* promoter activity in strains defective in YtfE, nitrate, nitrite and NO reductases and Hcp

The *ytfE* mutant strain, JCB5280 (red), and the *ytfE*$^{+}$strain JCB5270 (blue) were transformed with pNF383. The strains were grown anaerobically in MS either unsupplemented (pale red and pale blue bars) or supplemented with 0.5 of sodium nitrite (dark red and dark blue) and then sampled and assayed for β-galactosidase activity as described in Figure 3.1. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation).*,**,*** represents significance (P<0.05), (P<0.01) and (P<0.005) respectively.

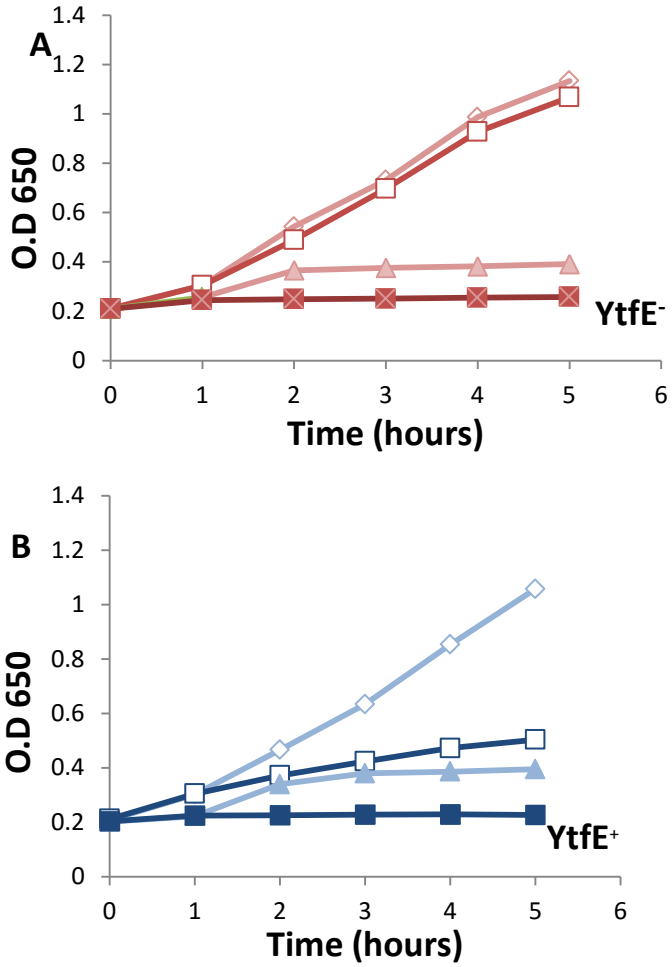

Figure 4.14:: Inhibition of growth by mulicopy *ytfE*$^{+}$ plasmid even in the absence of nitrosative stress

The YtfE^{+} strain, JC5270 (blue), and YtfE^{-} strain, JCB5280 (red), either were transformed with *ytfE*$^{+}$ plasmid (pACYC184) expressing *ytfE* gene (closed symbols) or were left untransformed as controls (open symbols). Both strains were grown anaerobically in MS in the presence of 1 mM sodium nitrite (dark blue closed and dark blue open symbols, the dark red closed and dark red open symbols) or in the absence of nitrite (pale blue closed and pale blue open symbols, pale red closed and pale red open symbols). The optical density was measured every hour for 7 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

This equivocal result could have been due either to extreme toxicity of YtfE itself, or to the stress of high level recombinant protein production. In order to resolve this uncertainty, both strains were retransformed with pET-24a::*ytfE*$^{+}$. Although high level expression of genes cloned into this vector requires both IPTG and the bacteriophage T7 RNA polymerase gene, pET vectors are sufficiently leaky to allow low level transcription of cloned genes even in hosts that lack the T7 RNA polymerase gene. Plasmid pET-24a::*ytfE*$^{+}$was therefore transformed into both strain JCB5270 and JCB5280 and the purified transformants were then grown anaerobically with or without 1 mM nitrite. The presence of the plasmid had virtually no effect on growth of the *ytfE*$^{+}$ host, JCB5270, in the presence or absence of nitrite (Figure 4.15, A). However, nitrite suppressed growth of the JCB5280 transformant, which grew well in the absence of nitrite (Figure 4.15, B). This again confirms that YtfE is generating NO from damaged metalloproteins that suppress growth.

4.11 Additional sources of NO that remain to be identified

Many publications have reported that *E. coli* is one of the many bacteria that contain molybdoproteins such as nitrate reductase (Taniguchi and Itagaki, 1960; Grove *et al*., 1996; Cole, 1996; Thomas *et al*., 1999; Johnson *et al*., 1991; Rajagopalan and Johnson, 1992; Sigel and Sigel, 2002; Magalon *et al.*, 2011; Hille *et al*., 2011). While other studies showed that tungstate, which is analogous to molybdate, is a competitive inhibitor of molybdenum uptake by bacteria and plants, and hence inhibits formation of molybdoenzymes (Diplock., 1971; Notton and Hewitt, 1971; Campbell *et al*., 1985; Deng *et al.*, 1989). First, in this study, to confirm that tungstate is able to inhibit the production of molybdate-dependent enzyme in *E. coli*, the parental strain, RK4353, was transformed with pNF383 plasmid and transformants were grown anaerobically in molybdate-deficient minimal salts medium and in normal minimal salts medium in the presence and absence of 2.5 mM nitrite.

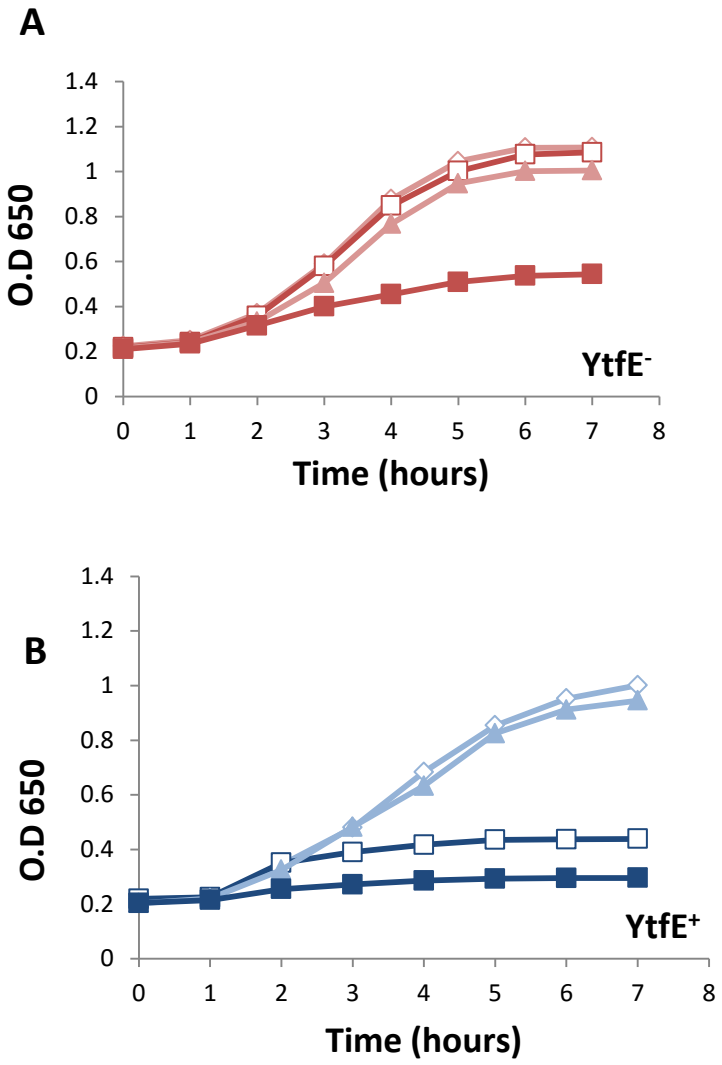

Figure 4.15: Growth and complementation of the *ytfE*$^+$strain, JCB5270 , and its *ytfE* derivative strain, JCB5280, with a plasmid pET24*:: ytfE*$^+$

Both strains JCB5270 (blue) and JCB5280 (red) were either transformed with plasmid pET-24a::*ytfE*$^+$(closed symbols) or were left untransformed control (open symbols). Both strains were grown anaerobically in MS in the presence of 1 mM sodium nitrite (the dark blue closed and dark blue open symbols, the dark red closed and dark red open symbols) or in the absence of nitrite (the pale blue closed and pale blue open symbols, the pale red closed and pale red open symbols). The optical density was measured every hour for 7 hours. Data from a typical biological replicate completed on different days and with different batches of medium.

Then, sodium tungstate (2 mM) was added to two cultures without molybdate while the other two with molybdate were left as unsupplemented controls. Samples (1 ml) were lysed and assayed for β-galactosidase activity just before nitrite addition, and then after 1 and 2 hours of anaerobic incubation. After two hours, the promoter activity of the cultures grown in the presence of molybdenum and nitrite in the absence of tungstate was significantly higher than the activity in cultures supplemented with tungstate and nitrite in the absence of molybdate ($p=0.0072$) (Figure 4.16). These data suggested that tungstate inhibits the production of molybdoenzymes by competing with molybdenum to bind to the active site of molybdoproteins and prevents the generation of NO from nitrite.

It has been previously demonstrated in this study that although deletion of genes for the two membrane-associate nitrate reductases, NarG and NarZ, resulted in greater resistance to nitrosative stress, some NO was still generated by the strain JCB5270, as shown by sensitivity to high concentrations of nitrite or NO (Figure 4. 11). Moreover, nitrite induction in the *ytfE*$^+$ strain elucidated that some NO was still being accumulated in the cytoplasm, even in the absence of all nitrate and nitrite reductases (Figure 4.13). One possible explanation for NO accumulation in this strain is that NO can be generated by one or more molybdoproteins other than two membrane-associated nitrate reductases, NarG and NarZ. If so, β-galactosidase activities should be lower in culture of strain JCB5270 after growth in the presence of tungstate. To confirm this prediction, the *ytfE*$^+$ strain, JCB5270, was transformed with pNF383 and transformants were grown anaerobically in molybdate-deficient-minimal salts medium and normal minimal salts medium in the presence and absence of 0.5 mM nitrite. Then, sodium tungstate (2 mM) was added to two cultures without molybdate while the other two with molybdate were left as unsupplemented controls as described previously. Samples (1 ml) were lysed and assayed for β-galactosidase activity just before nitrite addition, and then

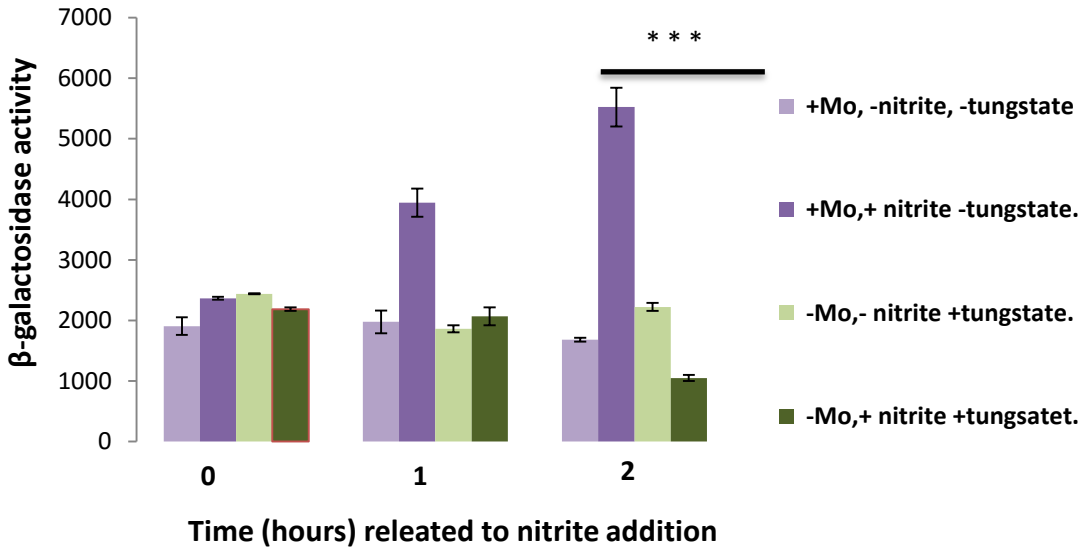

Figure 4.16: The effect of tungstate on NO generation from molybdate-dependent enzyme

The parental strain, RK4353, was transformed with the pNF383. The purified transformants were grown in minimal salts medium in the presence and absence of molybdate. One culture (dark purple) was pulsed with molybdate and 2.5 mM nitrite in the absence of tungstate while a control culture (pale purple without nitrite) was left as control cultures. The second group of cultures, one culture (dark green) was pulsed with 2.5 mM nitrite and 2 mM tungstate in the absence of molybdate while the control culture (pale green) without nitrite was left as control. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation). *,**,*** represents significance ($P<0.05$), ($P<0.01$) and ($P<0.005$) respectively .

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after 2 and 4 hours. Transcription at the *hcp* promoter after 4 hours was higher in the presence of nitrite and molybdate in the absence of tungstate, but lower when the cultures were supplemented with nitrite and tungstate in the absence of molybdate. The latter cultures grew at about half the rate of the unsupplemented control (Figure 4.17). As predicted, lower activities were found in the cultures supplemented with tungstate compared to the cultures supplemented with molybdate and this decrease was statistically significant (p=0.031). Again, the low expression at the *hcp* promoter suggested that tungstate is competing with molybdenum to bind to the active site of molybdoproteins other than NarG and inhibit formation of molybdoenzymes, and hence prevents generation of NO from nitrite.

4.12 Construction of a *mobA* mutant

The data presented above show that when the *ytfE*$^{+}$strain JCB5270 was grown under anoxic conditions in the presence of 1 mM of nitrite, growth is inhibited. This is consistent with nitrite being converted to NO even in the absence of the nitrate reductase, NarG. If there is another molybdoprotein that converts nitrite to NO, deletion of the *mobA* gene would prevent generation of NO and thus enable the mutant to grow in the presence of nitrite. To test this hypothesis, P1 transduction was used to transduce the *mobA* deletion from strain BW25113 *mobA::kan* to the *ytfE*$^{+}$ strain, JCB5270. The detection of the presence of the kanamycin resistance cassette (*kan*) in place of the *mobA* gene was confirmed by testing purified kanamycin resistant colonies by PCR with two primers, one that binds upstream and the other that binds downstream of *mobA*. Agarose gel electrophoresis was used to visualize the amplified PCR products (Figure 4.18). The transductants contained the correct kanamycin resistance cassette in place of *mobA* to produce new strain, RK4353 Δ*narZ* Δ*narGHJI* Δ*nrfAB* Δ*hmp* Δ*norV* Δ*nirBDC* Δ*hcp* Δ*mob::kan,* which was designated strain JCB52701.

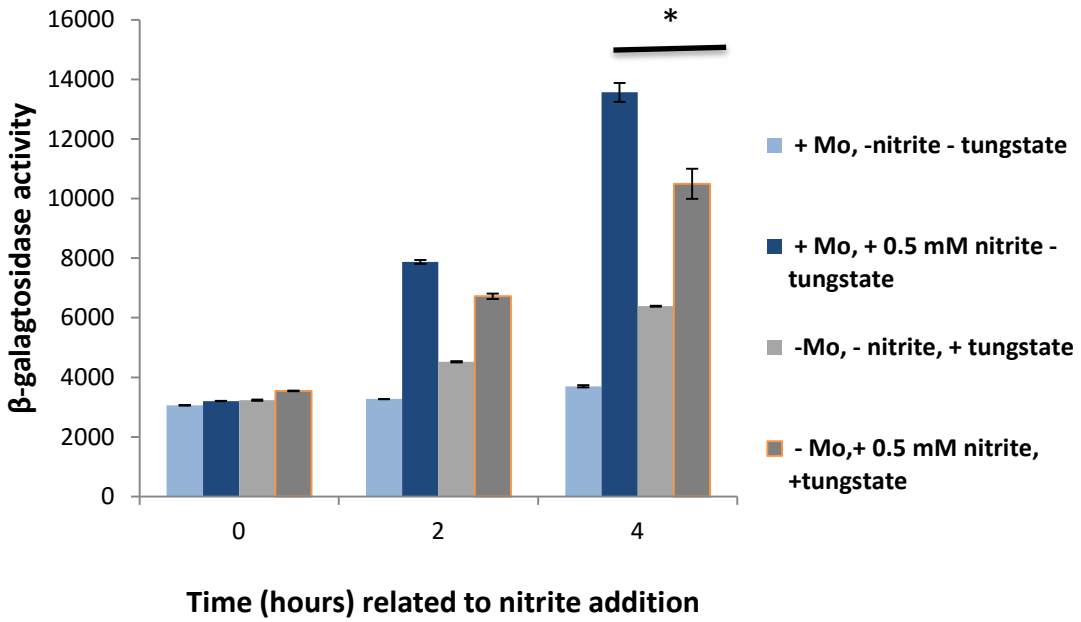

Figure 4.17: Effect of tungstate on NO production even in a strain that lacks NarG

The *ytfE*$^{+}$ strain, JCB5270, was transformed with the pNF383 plasmid. The purified transformants were grown in minimal salts medium in the presence and absence of molybdate. One culture (dark blue) was pulsed with molybdate and 0.5 mM nitrite in the absence of tungstate while the other culture (pale blue) without nitrite was left as control. For the second group of cultures, one culture (dark grey) was pulsed with 0.5 mM nitrite and 2 mM tungstate in the absence of molybdate, while the other culture (pale grey) without nitrite was left as control. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation). Other details are as in Figure 4. 8. *,**,*** represents significance (P<0.05), (P<0.01) and (P<0.005) respectively.

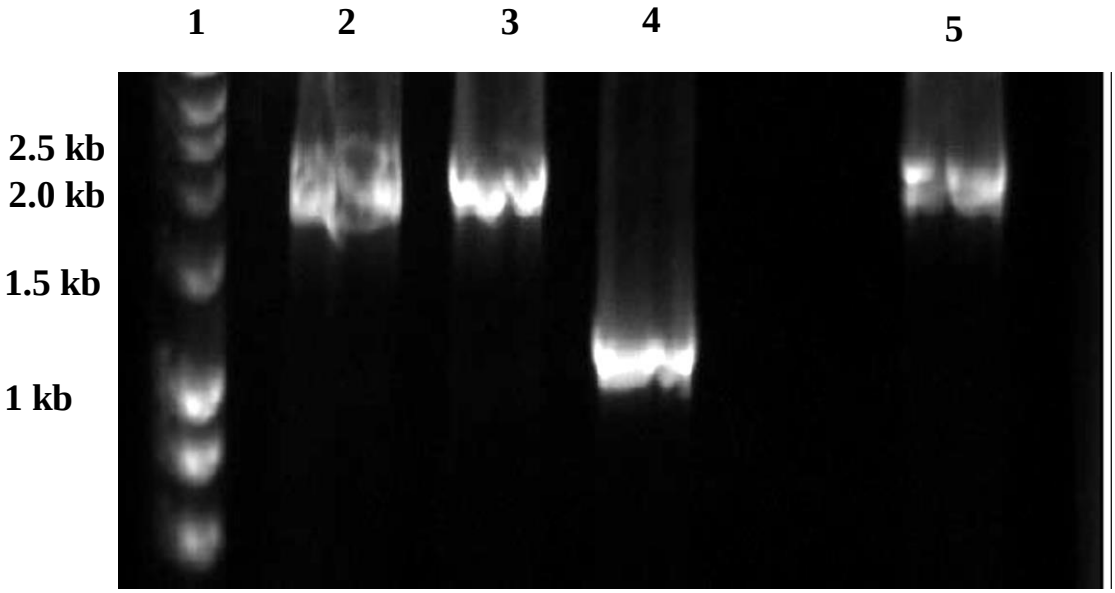

Figure 4.18: PCR analysis for the presence of the kanamycin resistance cassette or the *mobA* gene in strains BW25113 *mobA::kan*, JCB52701, and JCB5270

The *mobA* mutation was transferred using P1 transduction from BW25113 *mobA::kan* strain into *narZ narGHJI nrfAB hmp norV nirBDC hcp* strain, JCB2570, to generate *narZ narGHJI nrfAB hmp norV nirBDC hcp mobA* strain, JCB52701. Transductants and the recipient strain were grown on agar plates and tested by colony PCR for the presence of the *mobA* gene using primers *mobA* forward and *mobA* reverse. **Lane 1**: 1kb DNA marker (bands size are shown to the left), **Lane 2** and **3**: two different transductants candidates. **Lane 4**: JCB5270 as a template (Recipient), **Lane 5**: BW25113 *mobA::kan* strain as a template (Donor).

4.13 Effect of deletion of *mobA* on anaerobic growth during nitrite stress

To test whether molybdoproteins other than NarG also generate nitric oxide, growth of strains JCB5270 and its *mobA* derivative strain, JCB52701, in the presence and absence of 1 mM nitrite was compared. Both grew very well in the absence of nitrite, but growth of both strains was still inhibited by nitrite (Figure 4.19). These data therefore provided clear evidence that even in the absence of all molybdoproteins, NO is still generated from an unknown source and inhibits the growth of the *mobA* mutant.

4.14 Effect of deleting *mobA* on NO-dependent activation of the *hcp* promoter

In this study, it has been found that the growth in the presence of tungstate inhibits the production of NO from nitrite. In order to investigate whether deletion of the *mobA* gene would influence transcription at the *hcp* promoter in response to NO generated from nitrite, strains JCB5270 (*mobA*$^{+}$) and its *mobA* derivative strain, JCB52701, were transformed with the *hcp::lacZ* reporter plasmid. Transformants were grown anaerobically in minimal medium supplemented with 100% LB medium, 0.4% (v/v) glycerol, 0.4% (w/v) glucose and 20 mM fumarate in the presence and absence of 0.5 mM nitrite.

The activity of the *hcp* promoter in response to nitrite addition was slightly lower in the *ΔmobA* strain than in the *mobA*$^{+}$ strain, and this decrease was statistically significant (p=0.007) (Figure 4.20). However, as nitrite still stimulated *hcp::lacZ* expression there must be an additional source of cytoplasmic NO that cannot be due to NrfAB or a molybdoprotein.

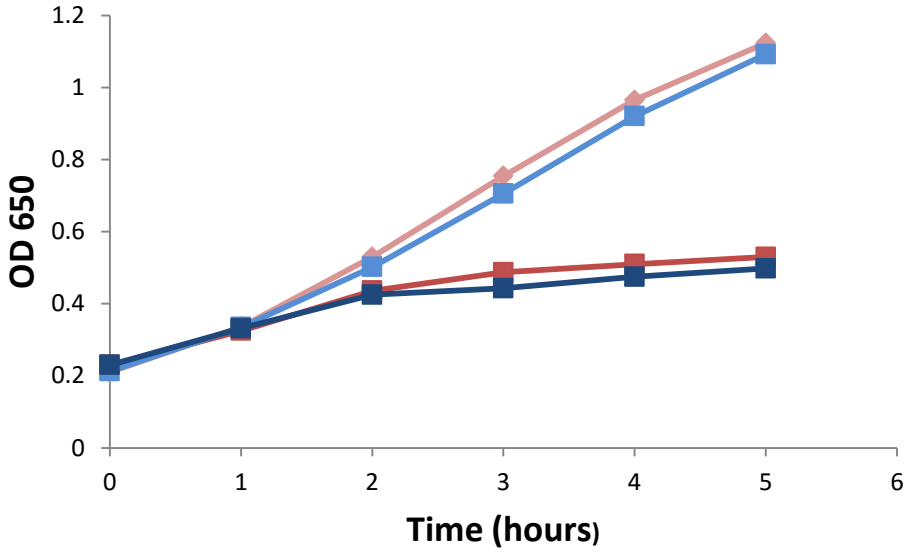

Figure 4.19: The effect of a *mobA* mutation on anaerobic growth in the presence and absence of nitrite

The strains JCB5270 (mobA$^+$) (red), and JCB52701 (mobA$^-$) (blue) were grown under anaerobic conditions at 37ºC without shaking in the absence of nitrite (pale red and pale blue lines) or with 1 mM nitrite (dark red and dark blue lines). The optical density for each culture was measured every hour for 5 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium. Other details are as in Figure 4. 1.

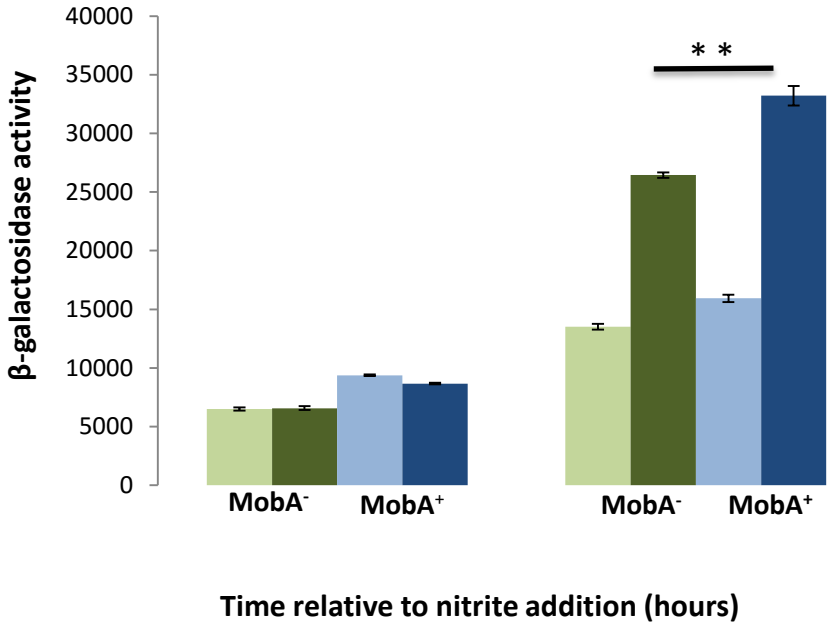

Figure 4.20: Effect of *mobA* mutation on transcription at the *hcp* promoter

The JCB5270 strain (*mobA*$^{+}$) (blue) and its *mobA* derivative strain, JCB52701 (green) were grown under anaerobic conditions in MS medium. One culture of each strain (dark blue and dark green bars) was pulsed with 0.5 mM nitrite while the other was an unsupplemented control (pale blue and pale green bars). Cell were lysed and assayed for β-galactosidase activity. Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation). Other details are as in Figure 3.1.*,**,*** represents significance (P<0.05), (P<0.01) and (P<0.005) respectively.

4.15 Complementation of the toxicity of YtfE in all nitrate, nitrite, NO reductases and *hcp* by a plasmid expressing the NO reductase, NorV

E. coli flavorubredoxin, NorV, is one of the protection mechanisms that are able to reduce NO to (N_2O) (Tucker *et al.,* 2005). The activation of transcription of *norV* by the nitric oxide sensor, NorR, is induced anaerobically under conditions of nitrosative stress (Gardener *et al.,* 2003; Justino *et al*., 2005). NorV participates in *E. coli* survival at all stages of macrophage infection (Baptista *et al*., 2012).

If the sensitivity to nitrosative stress of a $YtfE^+$ strain that lacks the high affinity Hcp-Hcr NO reduction system is due to the release of NO into the cytoplasm, toxicity should be relieved by complementation with a plasmid expressing the NO reductase, NorV. To test this prediction, the *ytfE*$^+$ strain JCB5270 and its *ytfE* derivative, strain JCB5280, were transformed with pAA182-PnorV. Transformants and untransformed control strains were grown in the minimal salts medium anaerobically in the presence or absence of either 1 mM nitrite or 5 μM NO, which was added as previously described. The cultures in which plasmid was present were supplemented with ampicillin. The optical density of transformants and untransformed strains were measured every hour for 5 hours. Both nitrite and NO strongly inhibited growth of the untransformed *ytfE*$^+$ strain (Figure 4.21, B), but almost had no effect on the growth of the NorV transformant (Figure 4.21, B).

As shown above, the *ytfE* deletion mutant was insensitive to 1 mM nitrite, but significantly was still sensitive to 5 μM NO. (Figure 4.21, A). Sensitivity to NO was again completely complemented by transformation with the *norV*$^+$ plasmid. These results convincingly demonstrated that YtfE releases, directly or indirectly, NO into the cytoplasm,

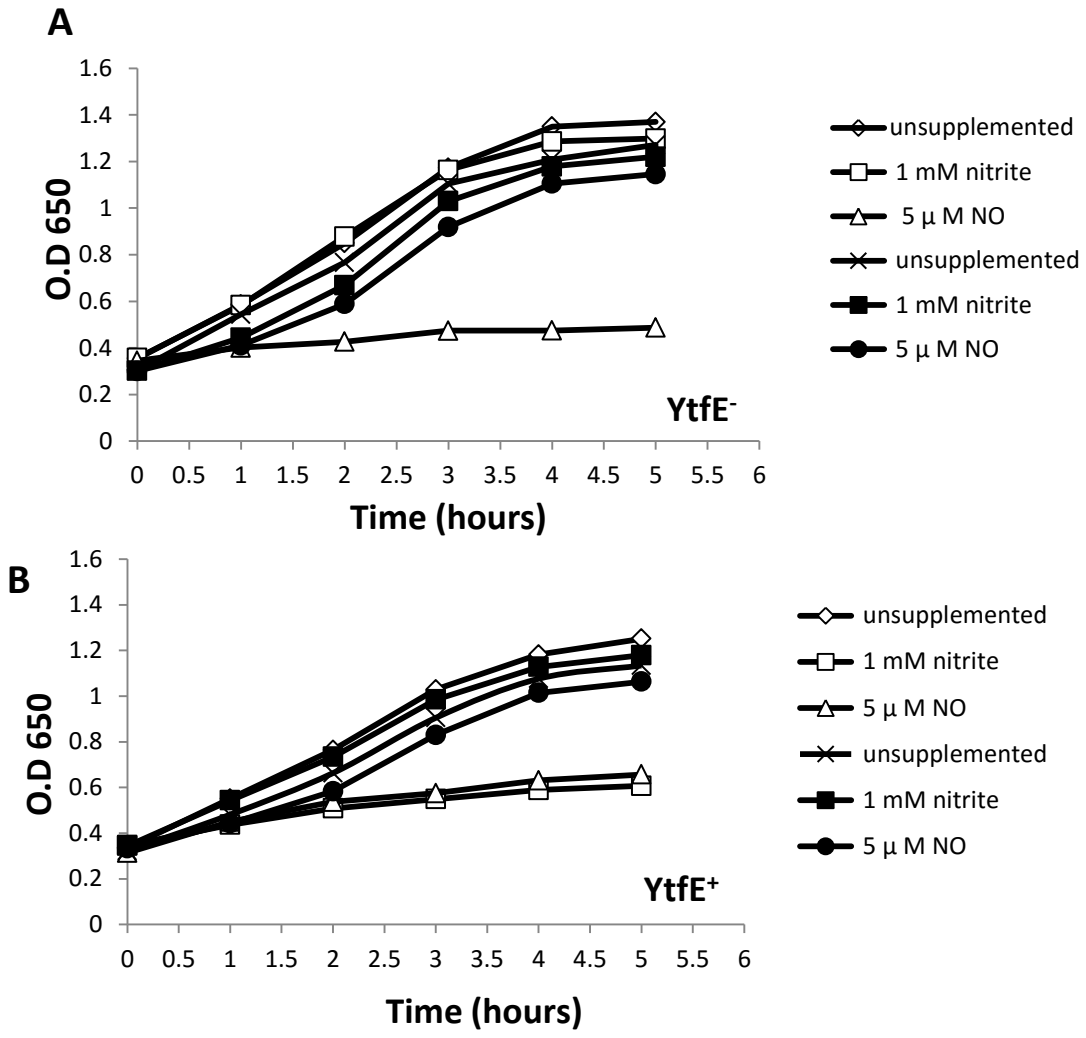

Figure 4.21: Complementation of the *ytfE*$^+$ strain, JCB2570, and its *ytfE* derivative strain by *norV*$^+$ plasmid

The strain *ytfE*, JCB5280, and strain, *ytfE*$^+$, JCB2570, were either transformed with the *norV*$^+$ plasmid, pAA182-PnorV, (closed symbols) or left as an untransformed control (open symbols). Bacteria were grown under anaerobic conditions at 37ºC without shaking in the presence or absence of 1 mM sodium nitrite or repeated additions of 1 µM or 5 µM of NO, in the presence of ampicillin to maintain the plasmid. The optical density of each culture at 650 nm was measured every hour for 5 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

and that either a functional Hcp-Hcr system or NorVW can prevent growth inhibition by the resulting nitrosative stress.

4.16 Complementation of a strain defective in all nitrate and nitrite reductases by an *hmp*$^+$ plasmid

In *E. coli*, synthesis of flavohaemoglobin, HmpA is increased in the presence of NO (Poole *et al*., 1996). During aerobic growth, Hmp is able to convert NO to nitrate (Hernandez-Urzua *et al*., 2003). However, under anaerobic conditions HmpA has the ability to reduce NO to N_2O (Corker and Pool, 2003; Pool, 2005), but the reduction activity is low and might not be enough to provide protection against endogenously generated NO from nitrite reduction (Gardner and Gardner, 2002). Work by Baptista *et al*. (2012) has shown that *E. coli* HmpA plays a critical role in protection during macrophage infection.

To resolve the controversy about whether Hmp can protect anaerobic cultures against nitrosative stress, strain JCB5270 was transformed with the plasmid pPL341 encoding Hmp and the original strain was used as an untransformed control. Transformants and untransformed control strains were grown anaerobically in minimal salts medium either unsupplemented or supplemented with 1 mM sodium nitrite. The transformed culture was supplemented with ampicillin to maintain the plasmid. The optical density for both cultures was monitored for 8 hours. Growth of the culture supplemented with sodium nitrite was almost completely complemented by the *hmp* plasmid when compared with the original strain, which was inhibited by nitrite (Figure 4.22). It was concluded that over-expression of HmpA from a plasmid is partially able to protect *E. coli* against nitrosative stress.

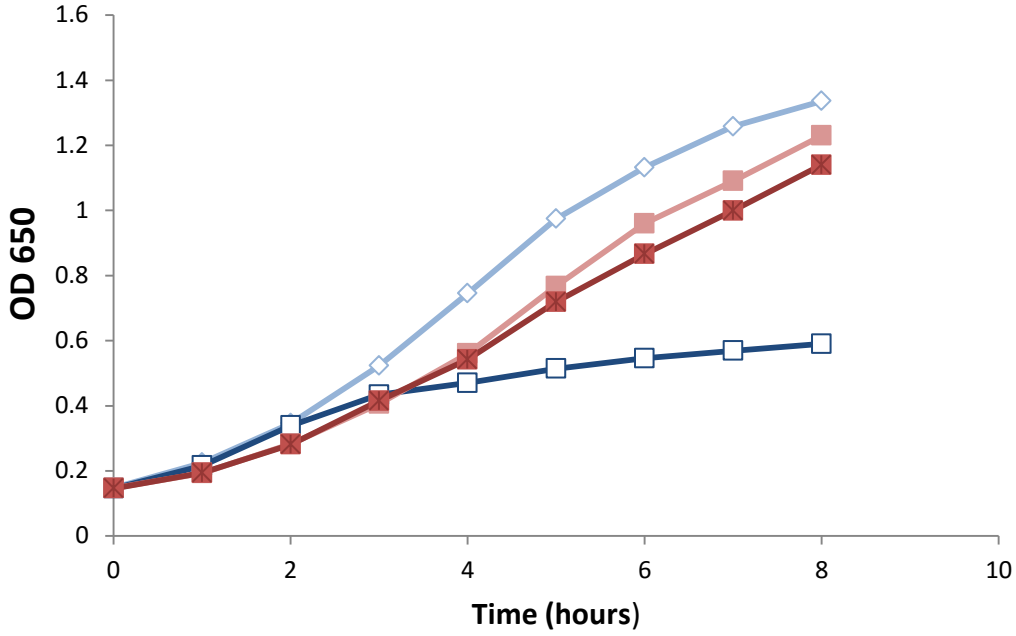

Figure 4.22: Complementation of the *ytfE*$^+$strain, JCB5270, that is defective in all nitrate and nitrite reductases with the *hmp*$^+$ plasmid

The *narZ narGHJI nrfAB hmp norV nirBDC hcp* strain, JCB2570, was transformed with the *hmp* plasmid, pPL341, (red closed symbols) or left as an untransformed control (blue open symbols). Bacteria were grown under anaerobic conditions at 37º C in the presence of 1 mM sodium nitrite (the open dark blue and closed dark red symbols) or in the absence of nitrite (the open pale blue and closed pale red symbols in the presence of ampicillin to maintain the plasmid. The optical density of each culture at 650 nm was measured every 8 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

4.17 Complementation of a strain defective in all nitrate and nitrite reductases by a *nrfA*$^+$ plasmid

The periplasmic enzyme, NrfA, of *E. coli* mediates the reduction of nitrite to ammonia and generates the toxic side product NO in oxygen limited environments. However, *in vitro* studies have shown that NrfA is also able to reduce NO to NH_4^+ (Poock *et al*., 2002; van Wonderen *et al*., 2008). In order to investigate whether NrfA is also able to complement the sensitivity of strain JCB5270 against the toxic nitric oxide, this strain was made competent and was transformed with the *nrfA*$^+$ plasmid (pLG339). The original strain was left as an untransformed control. Transformants and untransformed control strains were grown under anoxic conditions in minimal salts medium either unsupplemented or supplemented with 1 mM sodium nitrite. The transformed culture was supplemented with kanamycin to maintain the plasmid. Growth of the culture supplemented with sodium nitrite was partially restored by the *nrfA* plasmid when compared with the original strain **(**Figure 4.23). The results indicated that NrfA is partially able to prevent build-up of nitric oxide in the *E. coli* cytoplasm.

4.18 Implication of nitrite reductase, NirB, in NO accumulation in the *E. coli* cytoplasm

NirB is an NADH-dependent cytoplasmic nitrite reductase that reduces nitrite to ammonia during anaerobic growth. Synthesis of the NirB protein is induced by Fnr (Browning *et al*., 2005) and also by both NarL and NarP in the presence of high concentrations of nitrite or nitrate (Jayaraman *et al*., 1989; Tyson *et al.*, 1993). NirB has been characterised as a significant source of NO (Weiss, 2006). In order to confirm the results from previous studies that NirB also participates in the generation of NO, its effect on the

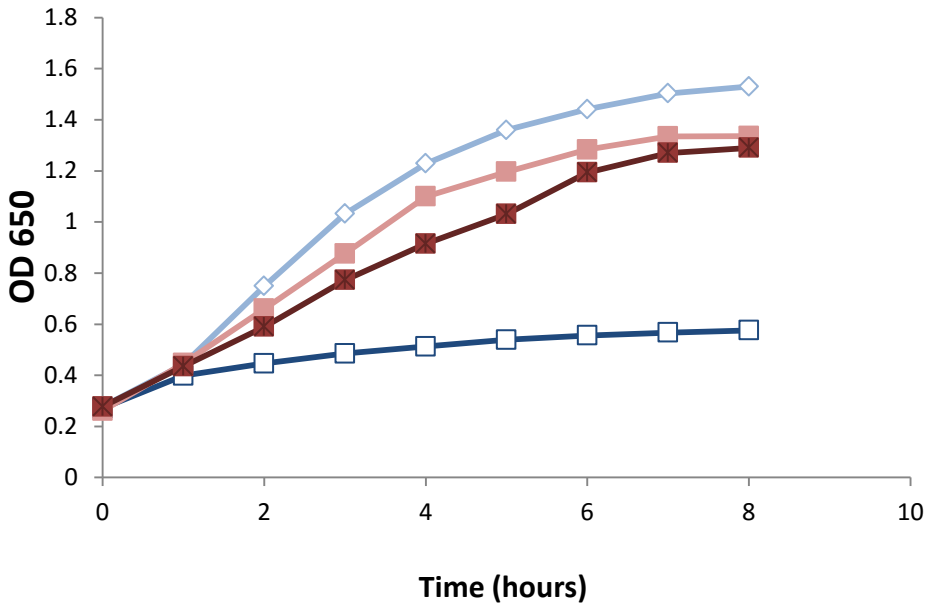

Figure 4.23: Complementation of the *ytfE*$^+$ strain, JCB5270, that is defective in all nitrate and nitrite reductases with a *nrfA*$^+$ plasmid

The *narZ narGHJI nrfAB hmp norV nirBDC hcp* strain, JCB2570, was transformed with the *nrfA*$^+$ plasmid, pLG339, (closed red symbols) or left as an untransformed control (open blue symbols). Bacteria were grown under anaerobic conditions at 37º C in the presence of 1 mM sodium nitrite (the open dark blue and closed dark red lines) or in the absence of nitrite (the open pale blue and closed pale red lines) in the presence of kanamycin to maintain the plasmid. The optical density of each culture at 650 nm was measured every hour for 8 hours. This graph shows data from a typical biological replicate completed on different days and with different batches of medium.

formation of NO in the cytoplasm was explored. For these experiments, an isogenic set of mutants was required, but the *nirB*$^{+}$ strain, JCB5256, was first transformed with pCP20 plasmid to remove its antibiotic resistance cassette to generate strain JCB5259. After curing with pCP20, P1 transduction was used to transfer the *ytfE* mutation from the RK4353 Δ*ytfE::cat* into the NirB^{-} strain, JCB5265, to generate strain JCB5272, and also into the NirB^{+} strain, JCB5259, to generate strain JCB5266.

Colony PCR was used to confirm that the transductants contained the correct chloramphenicol resistance cassette (*cat*) in place of the *ytfE* gene. After transduction with P1, the *nirB*$^{+}$ strain, JCB5266 and the isogenic *nirB* strain JCB5272 were transformed with plasmid PNF383. Both transformants were grown anaerobically in the presence or absence of 2.5 mM nitrite. The β-galactosidase activities of the cultures were determined after growth for 4 hours. Transcription at the *hcp* promoter was significantly higher in the isogenic *nirB*$^{+}$ strain in the presence of nitrite than in the *nirB* deletion mutant ($p=0.0081$) (Figure 4.24). These data suggested that less NO is produced in the strain defective in NirB, so the cytoplasmic nitrite reductase, NirBDC, is another protein responsible for generation of NO in the *E. coli* cytoplasm.

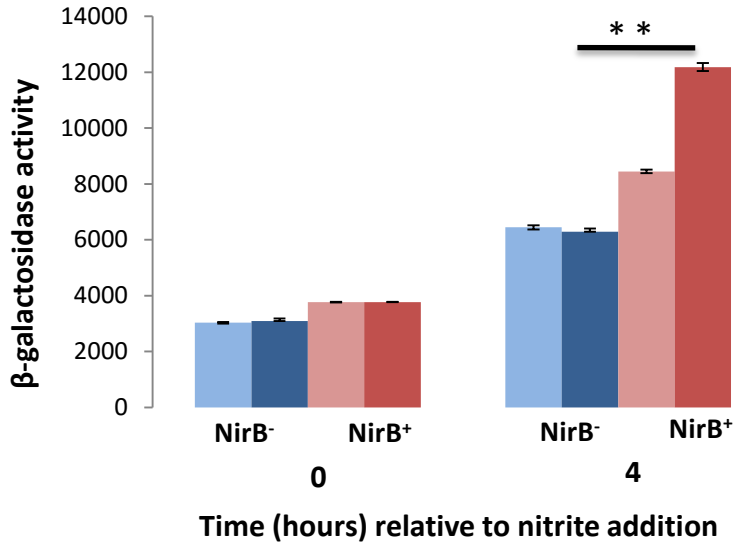

Figure 4.24: Nitric oxide generation by NirB protein in bacteria defective in nitrate, nitrite and NO reductases and the YtfE protein

The *nirB* strain, JCB5272, (blue) and the *nirB*$^{+}$ strain, JCB5266, (red) were transformed with the promoter reporter plasmid, pNF383. The strains were grown anaerobically in MS medium supplemented with TMAO and fumarate. One culture of each strain (blue and red bars) was pulsed with 2.5 mM nitrite while the other was an unsupplemented control (pale blue and pale red bars). Similar results were obtained in biological replicate experiments with independent cultures on different days, as shown by the error bars (mean +/- standard deviation). Other details are as in Figure 3.2. *,**,*** represents significance ($P<0.05$), ($P<0.01$) and ($P<0.005$) respectively.

4.19 Conclusion

Several conclusions can be drawn from the work presented in this chapter. First, neither Hcp nor YtfE is essential for anaerobic growth in the presence of nitrate or nitrite when other protective mechanisms against nitrosative stress such as NorVW, NrfAB and Hmp were functional. Aconitase and fumarase activities were also almost completely inhibited during anaerobic growth in media supplemented every 0.5 h with 1.5 μM NO, and this effect was largely dependent upon the absence of Hcp rather than YtfE.. Induction was restored in the *hcp ytfE* double mutant, strain JCB5228, because Hcp was no longer available to remove cytoplasmic NO generated by various mechanisms. YtfE is partially responsible for the extreme toxicity of NO by releasing it from nitrosylated proteins to a strain that lacks Hcp in addition to other known mechanisms of NO reduction. Therefore, YtfE is the main source of NO that has not been reported previously. Sensitivity to NO was completely complemented by transformation with the *norV*$^{+}$ plasmid. The presence of the pET-24a::*ytfE* plasmid had virtually no effect on growth of the *ytfE*$^{+}$ host, JCB5270, in the presence or absence of nitrite. In contrast, growth of the JCB5280 transformant was inhibited in the presence of nitrite, but not in its absence.

Although results presented in this chapter fully confirm the major role of NarG in reducing nitrite to NO, there are clearly other mechanisms for the production of cytoplasmic NO. Mutation of *moaB*, which is essential for synthesis of the active site of nitrite reduction by NarG, and also addition of tungstate to the growth medium both suppressed NO formation in the cytoplasm. However, in both cases, an NsrR-repressed promoter was still partially derepressed during anaerobic growth in the presence of nitrite.

CHAPTER FIVE-DISCUSSION

5.1 Regulation of the *hcp* promoter by the oxidative stress regulator OxyR

In *E. coli*, the transcription factor OxyR regulates activation of multiple antioxidant genes in response to oxidative stress, especially in the presence of H_2O_2 (Storz *et al*., 1990; Toledano *et al.,* 1994; Kullik *et al*., 1995). In the presence of H_2O_2 and S-nitrosothiols, OxyR controls transcription of many genes in order to protect against oxidative and nitrosative stress (Hausladen *et al*., 1996; Altuvia *et al*., 1997) and is itself quite sensitive to oxidative stress. Oxidation of one of six cysteine residue enables OxyR to induce transcription of genes involved in protection against oxidative stress (Kullik *et al.,* 1995). Based on microarray assays, OxyR activates expression of *katG*, which encodes Hydroperoxidase/Catalase I in the presence of S-nitrosocysteine (Hausladen *et al*., 1996). It has been claimed that S-nitrosylated OxyR regulates *hcp* transcription aerobically and anaerobically in the presence of nitrate, hence Hcp protects bacteria against endogenous nitrosative stress (Seth *et al*., 2012). The same group also found that *oxyR* mutant contains a higher amount of SNO-proteins than that of a parental strain and this generates nitrosative stress, which is improved by OxyR. Moreover, the *oxyR* mutant grew more slowly than the parental strain in the presence of nitrate. This impaired growth was rescued by using plasmid-overexpressed OxyR. The data of this group are consistent with the model presented in Figure 5.1, Model 1.

In the work presented in Chapter 3, it was clearly shown that the activity of the *hcp* promoter was low in both the parental strain, RK4353 and the *oxyR* mutant during aerobic respiration in the presence and absence of nitrate or nitrite (Figure 3.1). The growth phenotype of the *oxyR* mutant during anaerobic growth in the presence of nitrate or nitrite was

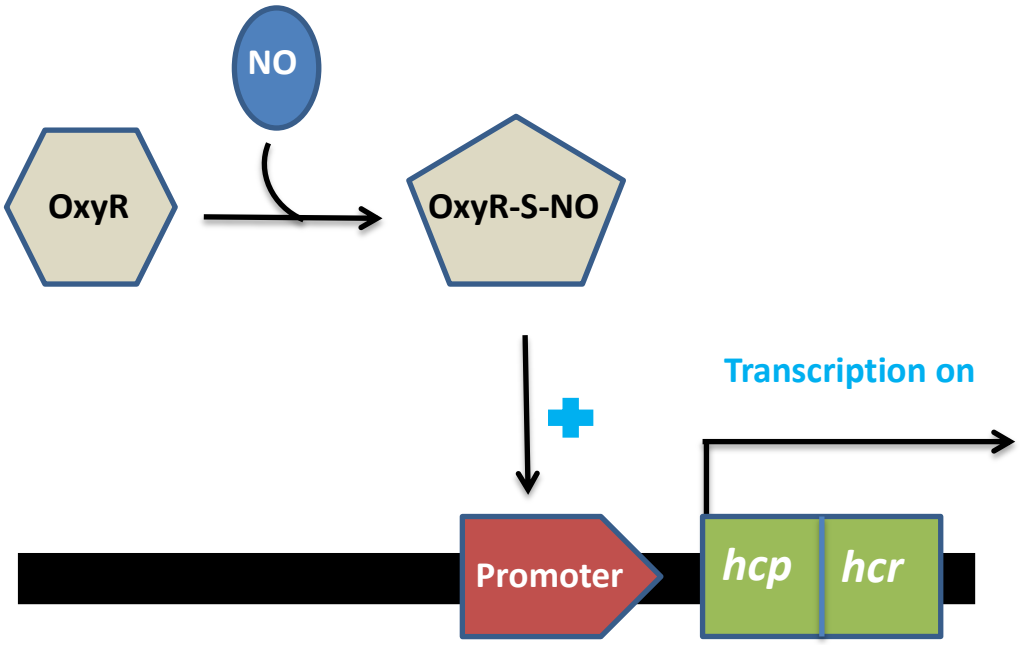

Figure 5.1: Model 1, Regulation of *hcp-hcr* transcription by nitrosylated OxyR in the presence of nitrosative stress

The model proposes that S-nitrosylated OxyR (grey) induces the transcription of the *hcp-hcr* (green) in order to protect bacteria against nitrosative stress according to Seth *et al*. (2012).

the same as that of the parental strain (Figure 3.2). Contrary to the report of Seth *et al.* (2012) the activity of the *hcp* promoter in an *oxyR* mutant was also similar to that of the parental strain under anaerobic conditions (Figure 3.3). During anaerobic respiration, growth of both *hcp fnr*$^+$ and *hcp*$^+$ *fnr* mutants were inhibited compared to that in the *hcp*$^+$ *fnr*$^+$ strain (Figure 3.5). The activity of the *hcp* promoter increased drastically in the *fnr*$^+$ strain in response to nitrite (Figure 3.6) or NO (Figure 3.7). However, only a low activity was detected in the *fnr* mutant in the presence of nitrite or NO.

The fact that an *oxyR* mutation had no effect on the activity of the *hcp* promoter during aerobic growth (Figure 3.1) was consistent with idea that in the absence of OxyR, ROS will accumulate and cause massive damage to FNR, converting it to inactive monomeric FNR. In this case, FNR is unable to bind DNA and thereby cannot activate *hcp* transcription (Figure 5.2, Model 2). However, the activity of the *hcp* promoter was high in the *oxyR* mutant under anaerobic conditions. Growth of the *oxyR* mutant was started at a high cell density (0.2) and then the culture was incubated for one hour for it to adapt properly to anaerobic conditions before addition of nitrate or nitrite. In this case, the [Fe-S] centre of FNR was reduced to the [4Fe-4S] centre, which in turn was able to bind DNA and thereby activate expression of *hcp* (Figure 3.3). These data are consistent with the Model 2 presented in Figure 5.2. In contrast, in the presence of OxyR (Figure 3.3), OxyR will remove ROS and therefore there is a little damage of FNR that might have happened during aerobic growth of the inoculum, and thereby it can activate transcription of the *phcp* (Figure 5.3, Model 3). In support of these results, Chismon *et al.* (2010) and Filenko *et al.* (2007) have shown that FNR anaerobically regulates the activity of the *hcp* promoter. Seth *et al*. (2012) concluded that S-nitrosylated OxyR induces the expression of the *hcp* promoter during both aerobic and anaerobic growth, in turn provides protection against nitrosative stress.

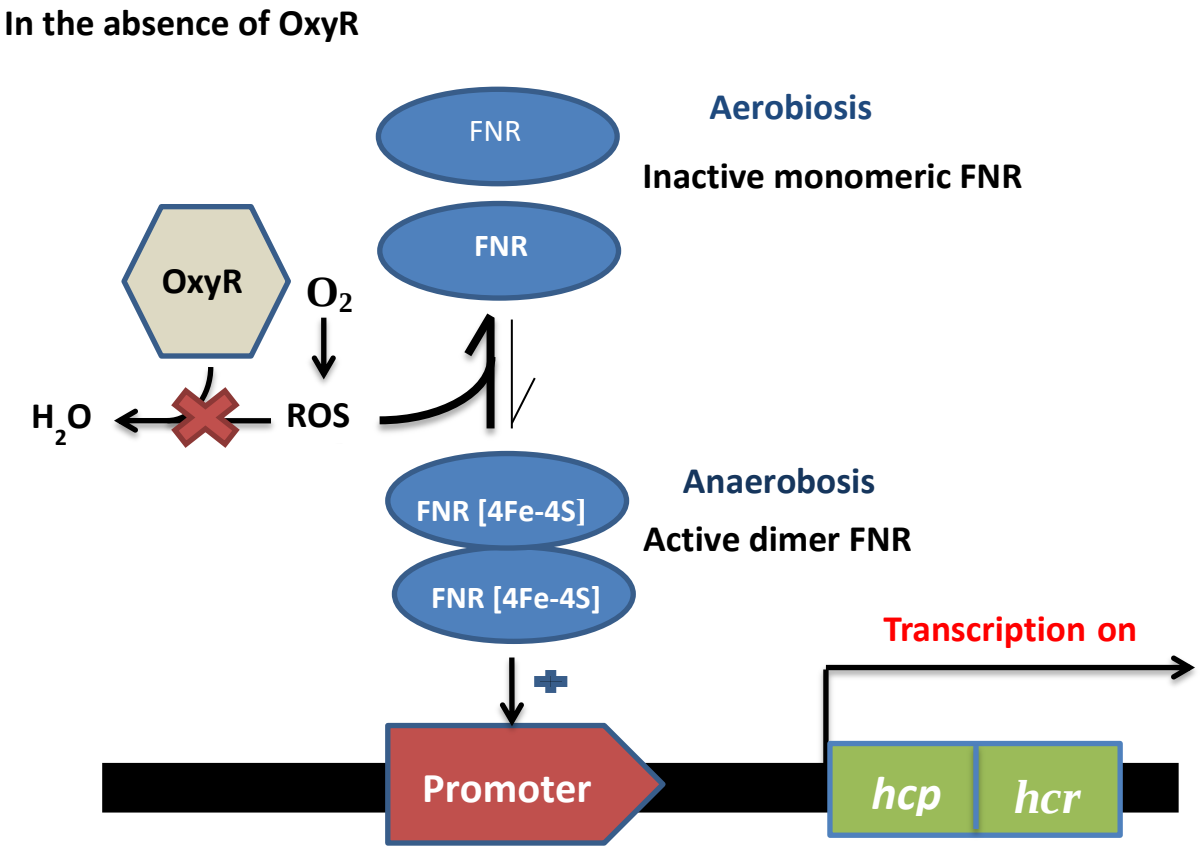

Figure 5.2: Model 2, The hypothesised mechanism of the lack of transcription activation of *hcp-hcr* by FNR during anaerobic growth in the presence of nitrosative stress

In the absence of OxyR (grey coloured) during aerobic conditions, FNR (blue coloured) cannot acquire its [4Fe-4S] cluster and thereby the *hcp-hcr* promoter cannot be activated, but during anaerobic conditions the FNR can acquire its [4Fe-4S] and activate the *hcp-hcr* promoter in order to protect bacteria against nitrosative stress.

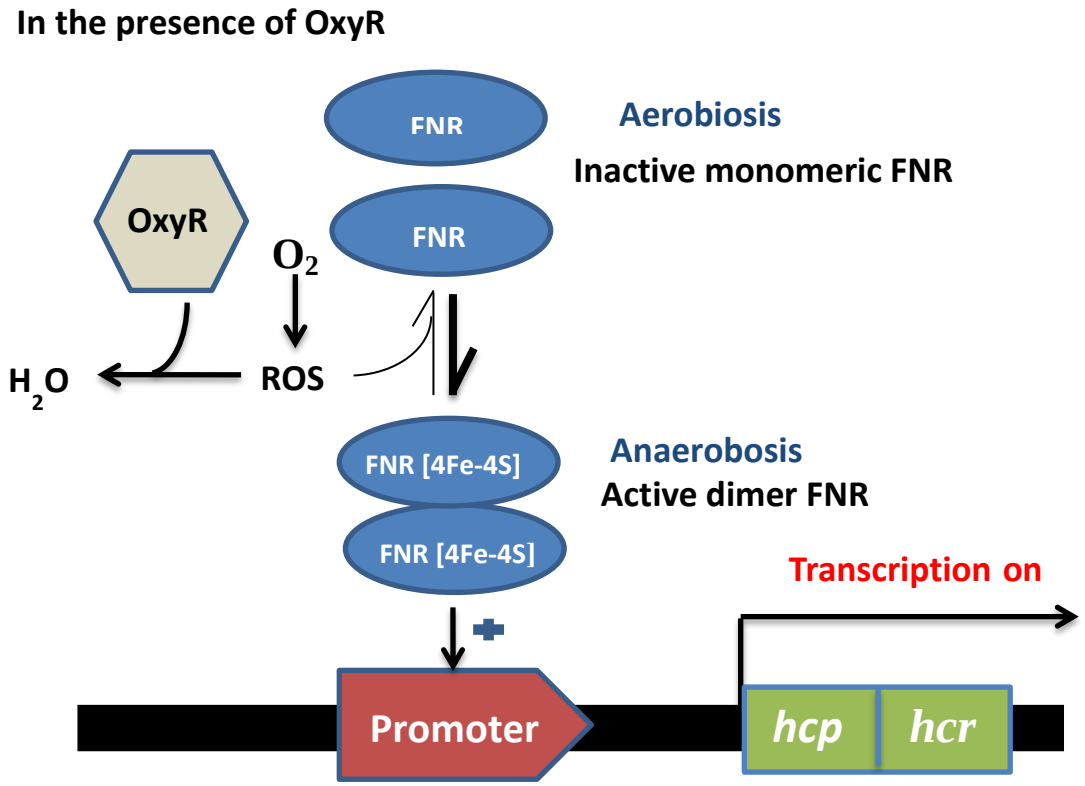

Figure 5.3: Model 3, The hypothesised mechanism of regulation of *hcp-hcr* by nitrosylated FNR during anaerobic growth in the presence of nitrosative stress

In the presence of OxyR (grey coloured), FNR (blue coloured) can acquire its [4Fe-4S] cluster and activate transcription at the *hcp-hcr* promoter in order to protect bacteria against nitrosative stress.

However, the results presented in Chapter 3 disagreed with this conclusion. The most likely explanation why an *oxyR* mutant is sensitive to nitrosative stress is that Hcp plays a critical role in protection. Because FNR is essential for *hcp* transcription, this requires both anaerobic growth and correct assembly of the iron-sulfur centre of the FNR protein. In an *oxyR* mutant, FNR might be damaged during aerobic growth of the inoculum. Furthermore, Seth and his group might have started their growth experiment at a low cell density, so adaptation to anaerobic growth was delayed. In summary, OxyR has no significant role in activation of the *hcp* promoter either aerobically or anaerobically to protect bacteria against nitrosative stress. However, FNR plays a key role in activation of transcription at the *hcp* promoter. Any effect of OxyR is therefore likely to be an indirect effect: in the absence of OxyR protein, FNR is inactive.

5.2 The role of YtfE in protection against oxidative stress under anaerobic conditions

Justino *et al*. (2007) suggested that YtfE might function as a protective protein against oxidative stress, as this protein is able to repair the damage to the iron-sulfur clusters of aconitase B and fumarase B caused by hydrogen peroxide. The same group also found that the activities of both enzymes were totally restored when purified holo-YtfE was introduced. In another study, it was suggested that YtfE is induced in oxygen-limited medium supplemented with H_2O_2 (Overton *et al.,* 2008). Results of the current study showed that there was no discernable difference in the anaerobic growth of four isogenic *ytfE* and *hcp* mutants in the presence and absence of H_2O_2 (Figure 3.4). This suggests that YtfE is not essential for protection against oxidative stress, so the results of this study were inconsistent with the conclusions of Justino *et al.* (2007) and Overton *et al*. (2008). In the previous studies, the

recovery of defective growth of the *ytfE* mutant in the presence of H_2O_2 might be due to another protein detoxification mechanism that protects bacteria against H_2O_2 toxicity. Data from previous studies and work presented in this study suggested that YtfE is more important in the presence of nitrosative stress conditions (Justino *et al*., 2005; Justino *et al*., 2006; Justino *et al*., 2007; Vine *et al*., 2010; Nobre *et al*., 2014).

5.3 Regulation of *nsrR* transcription during nitrosative stress

The transcription repressor, NsrR, operates in various pathogenic and non-pathogenic bacteria as a sensor of NO via an iron sulphur cluster. In the presence of NO, nitrosylation of its cluster can cause a loss of DNA binding activity (Tucker *et al.*, 2008, Tucker *et al.*, 2010). This relieves repression of NsrR target genes that are largely implicated in protection of *E. coli* against nitrosative stress.

In this project, the possibility that NsrR might autoregulate its own promoter in response to nitrite was investigated. If so, in the absence of NO, NsrR would supress the induction of NsrR, while in the presence of NO, the iron-sulfur clusters are nitrosylated and its transcriptional repression would be relived to allow transcription of the *nsrR* gene (Figure 5.4). However, the results of the experiments showed in two ways that the model in this figure is incorrect. First The β-galactosidase of the *nsrR::lacZ* fusion under aerobic and anaerobic conditions was low in the *nsrR* mutant in the presence and absence of the nitrite (Figure 3.9). Second, even in the absence of NsrR, only a low activity of *nsrR* promoter was detected and under the conditions of nitrosative stress, there was no induction (Figure 3.10).

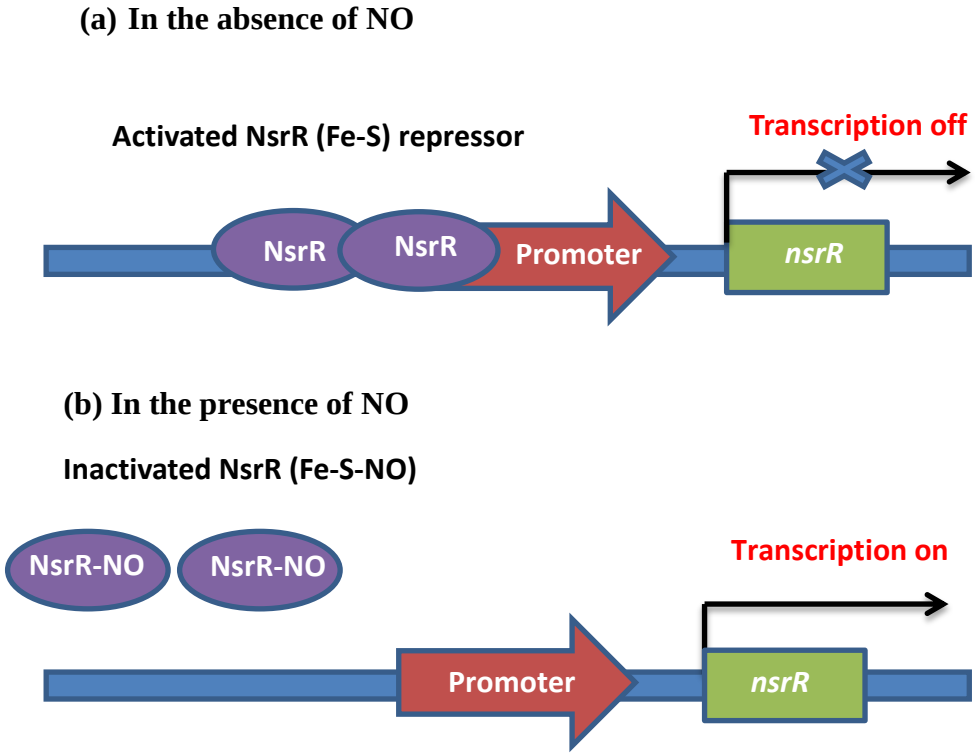

Figure 5.4: A model for the possible autoregulation of *nsrR* transcription by the NsrR protein and its response to NO

In the absence of the NO, NsrR is active as a repressor and prevents transcription of the *nsrR* gene. In the presence of NO, NsrR protein is nitrosylated and then it cannot bind to the promoter and hence transcription is on.

This result does not support the hypothesis concerning the function of the NsrR as auto-regulator of its own promoter. Thus, this study showed for the first time that the nitric oxide repressor protein, NsrR, does not regulate expression of the *nsrR* gene during aerobic or anaerobic growth. No evidence has yet been reported that NsrR is a transcriptional activator. It is possible that the *nsrR* promoter might be regulated by another transcription factor or its expression does not need any activator.

FNR is the master regulator for anaerobosis and activates transcription of many genes in order to protect the bacteria during nitrosative stress (Jayaraman *et al*., 1988). NsrR is the main sensor of NO under anaerobic conditions (Beaumont *et al*., 2004; Tucker *et al*., 2008; Tucker *et al*., 2010; Karlinsey *et al*., 2012) and plays a critical role in defence against nitrosative stress in *E. coli*. Both of these are vital for bacterial survival during anaerobic growth under these conditions. Hence, it is possible that FNR activates transcription of the *nsrR* promoter.

Therefore, in future experiments it would be interesting to test this hypothesis. This could be investigated using two strains. The fnr^+ strain and *fnr* mutant should be transformed with the *nsrR::lacZ* fusion reporter, and the β-galactosidase activity measured anaerobically in the presence of nitrite to determine whether the transcription activation of the *nsrR* gene is abolished in an *fnr* mutant. If the activity was low in the *fnr* mutant and high in the fnr^+ strain, it would be clear that FNR is responsible for the expression of the *nsrR* gene. However, if the activity was still low in both strains, it would be possible to design other future experiments to investigate whether there is another regulator of *nsrR* expression.

5.4 Minimal effect of an *nsrR* mutation on sensitivity to nitrosative stress

Three groups of experiments failed to yield evidence that NsrR fulfils an indirect role in protection against nitrosative stress. First, introduction of an *nsrR* mutation into the strain JCB5249 did not lead to any detectable increase or decrease in sensitivity to nitrosative stress (Figure 3.11, A, B), therefore loss of NsrR repressor function is not beneficial for growth under stress. Secondly, an isogenic set of *nsrR* and *hcp* derivatives of strain JCB5253 was used to show that, in contrast to loss of Hcp, loss of NsrR functions neither increased nor decreased sensitivity to nitrosative stress (Figure 3.12, A, B). Thirdly, the results showed that NsrR is not essential for activating expression of another NO reductase gene under conditions of nitrosative stress (Figure 3.13). These results are consistent with previous reports that concentrations of nitrite that are sufficient to generate growth-inhibiting concentrations of NO derepress *hcp* transcription by about 20% compared with an isogenic *nsr* deletion mutant (Vine *et al*., 2011). In *S. enterica*, NsrR repression was only partially relieved (10.2% compared with an *nsrR* deletion mutant) by 15 minute treatment with the NO-releasing agent, 100 μM diethylamine NONOate (Karlinsey *et al*., 2012). This implies that partial derepression of NsrR-regulated genes is sufficient to generate full protection against nitrosative stress. Furthermore, the data eliminated the possibility that loss of high affinity NO reduction in an *hcp* mutant might be an indirect effect of loss of an essential NO reductase encoded by an NsrR-activated gene.

5.5 The role of FNR in NO sensing

It has been reported that FNR is a physiological sensor of NO (Cruz-Ramos *et al*., 2002). This conclusion was reliant on clues that NO produced from proline NONOate can react with the iron-sulfur cluster of FNR to generate a mixture of monomeric or dimeric

DNIC. The exposure of bacteria to NO releasing compounds such as NOC 5 and NOC 7 for 5 minutes caused suppression of some gene expression but not the operons that are activated by FNR (Cruz-Ramos *et al.*, 2002). In the study by Pullan *et al.* (2007), deactivation of FNR by NO anaerobically activated some FNR-repressed genes including *ndh, hmp, gpmA* and *lpdA* and supressed FNR-activated genes such as *nrfA*. Consistent with deactivation of FNR by NO, Justino *et al.* (2005) also reported that during anaerobic growth in presence of NO, some FNR-activated genes are repressed, while FNR-repressed genes are induced. It has been shown that FNR represses transcription of the *hmp* gene that encodes the flavohaemoglobin, HmpA, but this repression can be negated by addition of NO (Poole *et al*., 1996; Gardner and Gardner, 2002). NsrR is the specific sensor of NO, which has been shown to negatively regulate the transcription of some genes implicated in the nitrosative stress response such as *hcp* (Filenko *et al*., 2007; Chismon, PhD Thesis, 2011) and *hmp* (Bodenmiller and Spiro, 2006; Filenko *et al.*, 2007; Rodionov *et al*., 2005).

Experiments designed to determine whether FNR is a physiologically relevant sensor that is inactivated by NO were based upon the fact that while FNR activates *hcp* transcription, it represses expression of *hmp.* Therefore, *hcp::lacZ* activity would decrease in response to FNR inactivation by NO, but *hmp::lacZ* activity would increase. In work presented in this study, the activities of the *hmp* and *hcp* promoters were much higher in the *nsrR* mutant strains both in the presence and absence of nitrite compared with that of the parental strain (Figure 3.14). Nitric oxide produced from nitrite reduction had no effect on FNR because the *hcp::lacZ* fusion promoter was active, as was the *hmp::lacZ* fusion promoter. This confirms that FNR was still active. Moreover, the activities of both promoters in the hcp^{+} strain and *hcp* strain were quite similar (Figure 3.13). Additionally derepression of *hcp::lacZ* and *hcp::lacZ* expression indicated that FNR was functional (Figure 3.13). These data strongly

suggest that FNR cannot play a physiologically significant role as a sensor of NO In agreement with these findings, other studies found that NO in the *E. coli* cytoplasm could not damage FNR sufficiently to function as a physiologically relevant sensor (Vine *et al*., 2011; Fleischhacker and Kiley, 2011).

The results of this study therefore contradict the claim that FNR can play a significant role as a physiological sensor of NO (Cruz-Ramos *et al*., 2002). However, there are three problems with these earlier experiments. First, the experiments were completed before the discovery of NsrR. It has been reported that in *Salmonella enterica*, NsrR can respond to a very low concentration of NO and thereby activate transcription of many genes involved in nitrosative stress response (Karlinsey *et al*., 2012). Secondly, a very high concentration of NO was used that was sufficient to damage the Fe-S centres of FNR chemically, but resulted in only a two-fold loss of FNR function. Thirdly, it was known that the concentration of NO used in these earlier experiments was 100-fold higher than [NO] that accumulates physiologically during anaerobic growth in the presence of nitrate or nitrite. Therefore the results of Chapter 3 concluded that derepression of *hmp* by accumulated NO was due to of NO-activated derepression by NsrR, but not by FNR.

5.6 The role of the YtfE (RIC) protein in the accumulation of nitric oxide in the *E. coli* cytoplasm under conditions of nitrosative stress

The product of the *E. coli ytfE* gene is a di-iron protein, YtfE (or RIC for the repair of iron-centres of proteins damaged by oxidative or nitrosative stress). YtfE has been extensively implicated in the repair of iron-sulfur centres of proteins damaged by nitrosation. This conclusion was dependent on evidence that YtfE plays a vital role in rendering resistance against NO (Justino *et al*., 2005). Growth of the *ytfE* mutant was very poor during anaerobic

growth under a range of conditions, and recombinant YtfE was unable to reduce NO (Justino *et al*., 2006). The activities of aconitase and fumarase had decreased anaerobically in the *ytfE* mutant in the presence of NO and H_2O_2 but the activities of both enzymes were restored *in vitro* when purified YtfE was introduced (Justino *et al*., 2007). Furthermore, the deletion of *ytfE* led to an increase of the intracellular free iron levels. It was concluded that the role of YtfE is not to repair iron-sulfur clusters that are damaged by NO but might be to recruit ferrous ions into the NO-damaged iron-sulfur clusters (Justino *et al*., 2007). Recent studies have shown that iron atoms can be transferred from RIC to iron-deficient iron-sulfur centres (Nobre *et al*., 2014). However, as the rate is extremely low, the possibility that YtfE catalyses another reaction was investigated**.** In the work presented in Chapter 4, the phenotype of an *hcp* and *ytfE* mutant in a background that lacks other known systems for NO reduction, NorVW, NrfAB, NirB and Hmp, was first completed. During anaerobic growth in the presence of NOSW, two main phenotypes were detected in each growth experiment containing all combinations of the presence or absence of *ytfE* and *hcp* mutations.

The results showed that growth of the $Hcp^{-}YtfE^{+}$ strain, JCB5250 (Figure 4.4, B) was completely abolished in the presence of 1.5 μM NO but a mutation in both *hcp* and *ytfE* resulted in a strain that was able to grow slowly (Figure 4.4, D). However, growth of the $Hcp^{+}YtfE^{+}$ and $Hcp^{+}YtfE^{-}$ strains was unaffected (Figure 4.4 A, C). It was concluded that YtfE, which was previously proposed to be a major factor in providing protection against nitrosative stress, had no detectable effect on the survival of the four strains exposed to nitrosative stress. However, YtfE is at least partially responsible for the release of NO into the cytoplasm of the strain that lacks Hcp in addition to other known mechanisms of NO reduction. In contrast, Hcp is the key player in providing this protection. This role of Hcp to confer this protection was correlated with our recent study that confirmed that Hcp plays an

essential role in a high affinity but low capacity nitric oxide reduction (Wang *et al.*, 2016; in submission). Several studies have previously shown that a major source of cytoplasmic NO is due to a side reaction catalysed by the cytoplasmic nitrate reductase, NarG, in which nitrite is reduced to NO (Calmels *et al.*, 1988; Ralt *et al*., 1988; Metheringham *et al*., 1994; Gilberthorpe and Poole 2006; Vine *et al.*, 2011; Seth *et al*., 2012). Some iron-sulphur proteins immediately trap this NO, resulting in the inactivation of key metabolic functions and growth inhibition (Seth *et al.*, 2012; Vine *et al.*, 2011). In this study, the results also have shown that NarG, along with YtfE, are responsible for generating NO, as the activity of the *hcp* promoter in a *napAB narG ytfE* strain was lower than that of the *narG napAB* mutant (Figure 4.9). Anaerobic growth of a $YtfE^{+}$ strain that lacks all known nitrate, nitrite and NO reductase activities, including the hybrid cluster protein, Hcp, is severely inhibited by nitrosative stress including nitrite (Figure 4.11, B) or NO (Figure 4.12, B) but was partially restored by further mutation of *ytfE* (Figure 4.11, A). Moreover, less derepression (2-fold) of *hcp::lacZ* transcription was found in a *ytfE* mutant than in the parent strain (Figure 4.13), again indicating that YtfE contributes significantly to the release of cytoplasmic NO.

Nitric oxide is a small radical species that is widely assumed to freely equilibrate across bacterial membranes. It has been found that although transcription at NsrR-regulated promoters was derepressed during growth in the presence of nitrite, high concentrations of NO were far less effective (Vine *et al*., 2010). Some *E. coli* iron-sulfur proteins are especially sensitive to inactivation even by low concentrations of NO. Inactivation of IlvD has been reported to be the primary reason for growth inhibition in minimal medium by NO (Gardner *et al* 1997; Justino *et al.*, 2007). However, other iron-sulfur enzymes such as aconitase B and fumarase B that play important roles in intermediary metabolism during anaerobic growth are also inactivated by NO (Ren *et al.*, 2008; Justino *et al*., 2007). To further investigate whether

the inactivation of these enzymes is due to NO released by YtfE from nitrosylated iron-sulfur proteins, the activities of aconitase and fumarase in the cultures of the four isogenic mutant strains were determined during nitrosative stress. Aconitase activity was almost undetectable after anaerobic growth of the two Hcp-deficient strains, JCB5250 and JCB5260, in the presence of 1.5 μM NO (Figure 4.6, A, B). Fumarase activity was also extremely low in the same strains after growth in cultures pulsed with NO (Figure 4.7, A, B). This effect was largely dependent upon the absence of Hcp rather than YtfE. In contrast, aconitase and fumarase activities were readily detectable in both of the *hcp*$^{+}$ strains, JCB5210 and JCB5257 (Figures 4.6, 4.7, A, B). In the YtfE^{-} strain, JCB5257, both enzymes were still active. This indicates that even if YtfE repairs damaged iron-sulfur proteins, the rate of the repair reaction is quite slow.

In *E. coli*, there are two distinct aconitases, AcnA and AcnB (Brock *et al*., 2002). The induction of AcnA is increased during oxidative stress, while supressed during anaerobic growth (Gruer and Guest, 1994). In contrast, expression of the AcnB enzyme is highly induced anaerobically during the exponential phase of growth (Cunningham *et al*., 1997; Gruer *et al*., 1997). Based on these previous reported observations, the activity that was assayed in this work was probably due to the aconitase B. Moreover, there are three fumarases in *E. coli*, two of which are iron-sulfur proteins. The residual activity in cultures subjected to nitrosative stress is possibly due to the third isozyme, FumC. These results suggested that YtfE does not repair proteins damaged by nitrosative stress by the mechanism previously reported, but instead it releases free NO from proteins damaged by nitrosylation. In addition to this, Hcp fulfils a major role in reducing the toxic product that is released by YtfE.

Other experiments provided evidence that YtfE is an enzyme that catalyses the release of nitric oxide from cytoplasmic proteins damaged by nitrosylation. Transformation of *ytfE*

mutant with a *ytfE*$^{+}$ expression plasmid during growth in the presence of nitrite restored extreme sensitivity to nitrosative stress, confirming that YtfE was directly responsible for the observed growth inhibition (Figure 4.15). However, complementation of the toxicity of YtfE in an *hcp* mutant by a plasmid expressing the NO reductase, NorV (Figure 4.21), convincingly confirmed that YtfE generates directly or indirectly NO in the *E. coli* cytoplasm and that either a functional Hcp-Hcr system or NorVW can prevent growth inhibition by the resulting nitrosative stress. In addition, complementation of the *ytfE* mutant, JCB5270, using an *hmp* plasmid (Figure 4.22) and *nrfA* plasmid (Figure 4.23) indicated that Hmp and NrfA are only partially able to prevent NO accumulation in the *E. coli* cytoplasm.

A recent study by Nobre *et al*. (2014) from the Justino research group showed that YtfE may interact with the side of IscS/SufS in order to donate iron and sulfur that are required to assemble iron-sulfur centres *in vivo*. These data are consistent with the model presented in Figure 5.5. In this case, this process will agree with the negative interaction that has been observed between AcnB and YtfE (Wang, PhD Thesis, 2015).

If YtfE is involved in the donation iron through the ISC system (Isc, Suf), instead of directly repairing damaged iron-sulfur clusters on its own, it would not be essential for YtfE to interact with iron-sulfur proteins such as aconitase and fumarase, but YtfE would only be required to interact with the Isc proteins. Therefore, in future experiments it would be interesting to test this hypothesis in a two-hybrid system to investigate whether YtfE is able to interact with any of the Isc proteins.

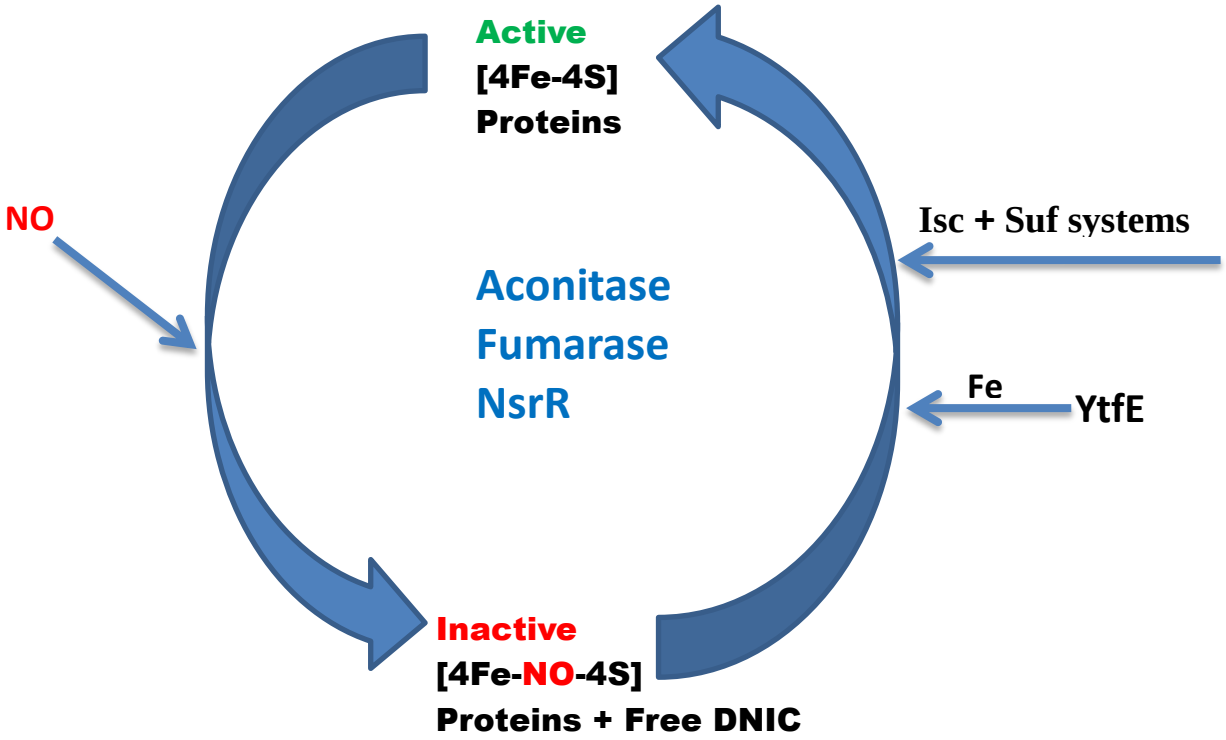

Figure 5.5: A model for the coordination of the YtfE and the Isc proteins in donation of iron for the generation of [Fe-S] clusters.

The model proposes that YtfE repairs proteins damaged by nitrosative stress by donating iron to the inactivated iron-sulfur proteins by removing the DNIC in the presence of Isc and Suf systems (Nobre *et al*., 2014).

Note, however, that all of the data presented in Chapter 4 contradicted previous studies that claimed to show that YtfE plays a key role in repairing damaged iron-sulfur proteins (Justino, *et al*., 2005; 2006; 2007; 2009; Nobre *et al.*, 2014; Overton *et al*., 2008).

The combined observations of this study led us to propose the model for how very low concentration of NO released into the *E. coli* cytoplasm are detoxified (Figure 5.6). The model proposes that NO binds rapidly to metalloproteins such as aconitase, fumarase, IlvD and probably many others, inactivating their metabolic functions. The function of YtfE is to reverse this initial damage, allowing bacterial growth to continue under conditions of mild nitrosative stress. According to this model, the role of Hcp is to reduce the resulting very low concentration of NO as rapidly as it is formed (Figure 5.6). In summary, YtfE and Hcp coordinate the repair of nitrosative damage and prevent NO accumulation. In the absence of YtfE, secondary reactions such as trans-nitrosylation of the –SH groups of proteins and peptides such as glutathione would result in damage that is more difficult to repair. This proposal is contrary to that proposed by other groups that YtfE repairs iron-sulfur centres by replacing iron atoms released during nitrosative stress.

5.7 Alternative sources of nitric oxide in *E. coli*

E. coli contains many other molybdoproteins in addition to nitrate reductase (Taniguch and Itagaki, 1960; Grove *et al*., 1996; Cole, 1996; Thomas *et al*., 1999; Johnson *et al*., 1991; Rajagopalan and Johnson, 1992; Sigel and Sigel, 2002; Magalon *et al.*, 2011; Hille *et al*., 2011). Although deletion of genes for the two membrane-associated nitrate reductases, NarG and NarZ, and also nitrite reductases NirB and NrfA resulted in greater resistance to nitrosative stress, some NO was still generated by strain JCB5270, as shown by sensitivity to 2011).

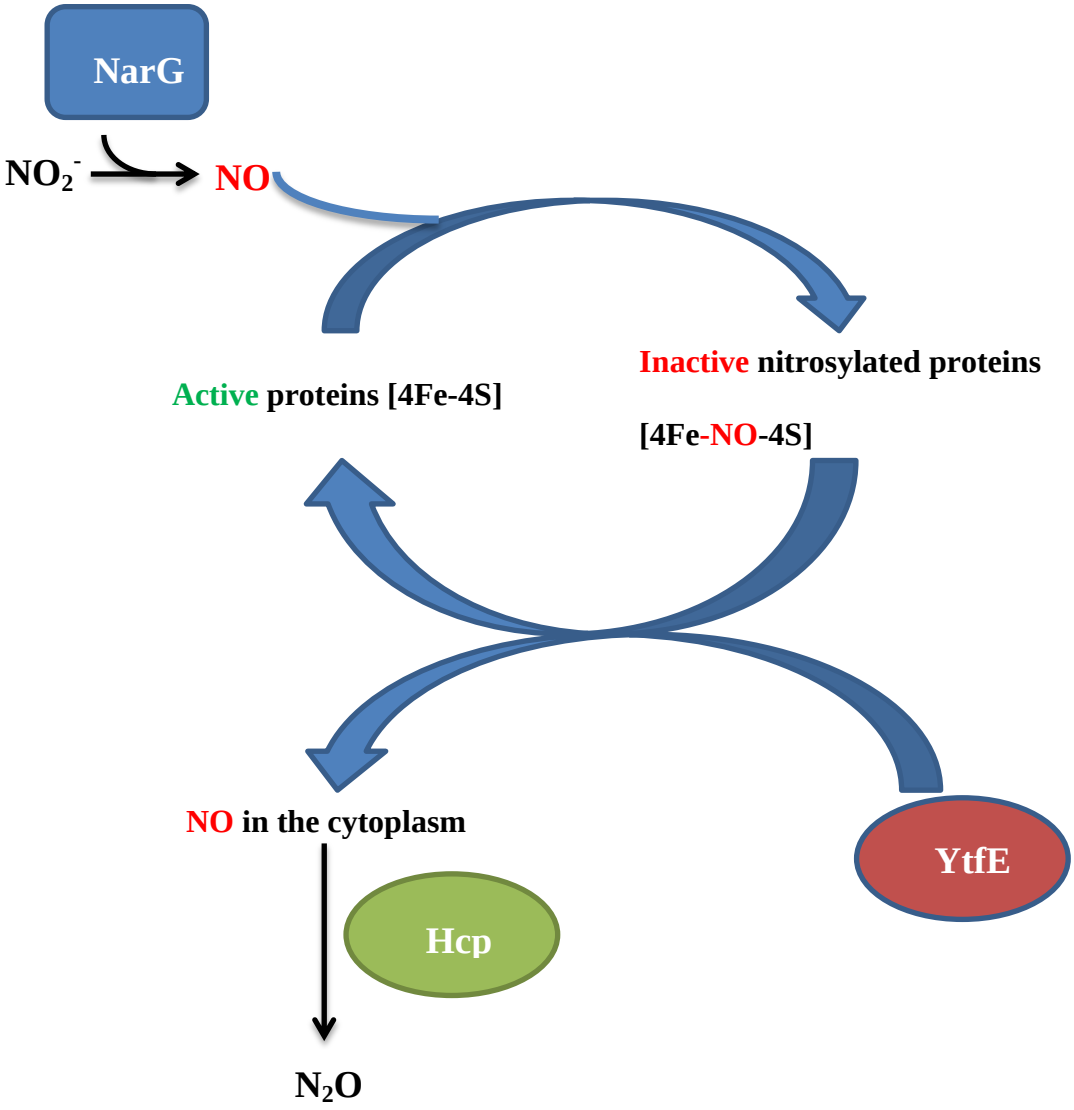

Figure 5.6: A model for the coordinated removal of NO from proteins inactivated by nitrosation

The model proposes that YtfE repairs proteins damaged by nitrosative stress by releasing NO from the damaged iron atoms of iron-sulfur proteins into the cytoplasm and that Hcp reduces these low levels of NO to the less toxic N_2O.

high concentrations of nitrite or NO (Figures, 4.11, 4.12). Nitric oxide production in the Δ*narG* Δ*narZ* mutant can be explained that NO might be generated by one or more molybdoproteins other than NarG and NarZ. It has been reported that tungstate, which is identical to molybdate, is a competitive inhibitor of molybdenum uptake by bacteria and thereby suppresses formation of molybdoenzymes (Diplock., 1971; Notton and Hewitt, 1971 Campbell *et al*., 1985; L'vov *et al.*, 2002; Hagen, 2011). For instance, both periplasmic nitrate reductases (Gates *et al*., 2003) and DMSO (Buc *et al*., 1999) from *E. coli* become inactive in the presence of tungstate rather than molybdenum. In the current work, lower activities were found in the cultures supplemented with tungstate, but some residual induction of *phcp* transcription was still detected (Figure 4.17). This lower expression at the *hcp* promoter suggested that tungstate is competing with molybdenum to bind to the active site of molybdoproteins other than NarG, and prevents generation of NO from nitrite. These data are consistent with the model presented in Figure 5.7.

Mutants defective in *mob* genes are unable to synthesise the molybdopterin guanine dinucleotide cofactor and are therefore defective in all molybdopterin-dependent activities. The β-galactosidase activity of a *mobA* derivative of strain JCB5270 was also lower than that of the *mob*$^{+}$ strain (Figure 4.20). However, the NsrR-repressed promoter was still partially depressed during anaerobic growth in the presence of nitrite. In conclusion, there are clearly other mechanisms for the production of cytoplasmic NO that cannot be due to NirBD, NrfAB or the molybdoproteins. Moreover, it has been found that *Bacillus vireti* generates NO from unknown source that can be reduced to nitrogen via nitrous oxide by using unusual two NO

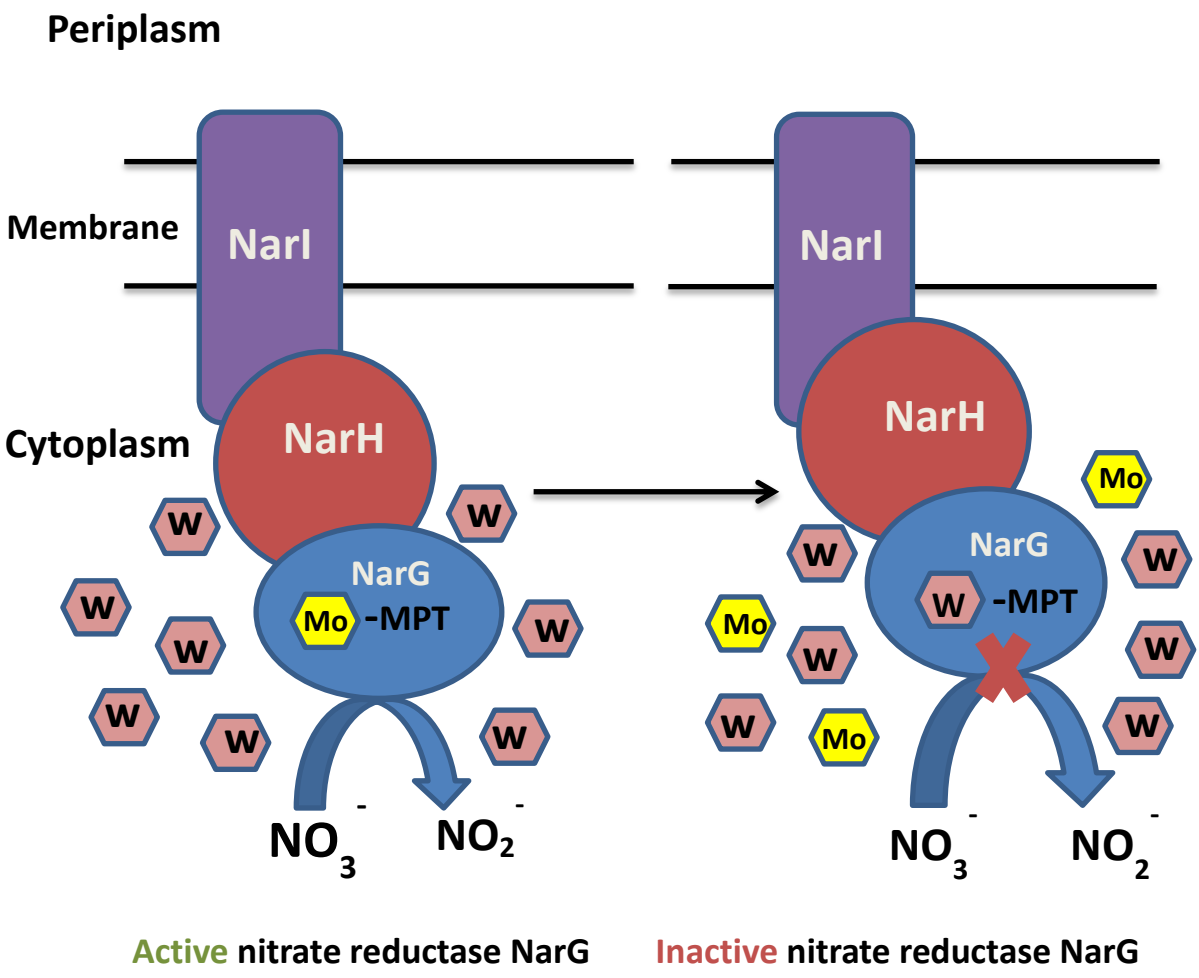

Figure 5.7: Comparison of tungstate and molybdate trace elements to compete for incorporation into nitrate reductase molybdenum cofactor (MoCo)

Tungstate (pink) competes with molybdate (yellow) for incorporation into the MoCo as **MPT,** which causes inactivation of nitrate reductase NarG (blue). Thus, the nitrate reductase is unable to generate NO from nitrite, or to convert nitrite into NO.

reductases including qCu_A Nor that encoded by *cbaA* and a z-type N_2O reductase encoded by *nosZ (*Mania *et al* ., 2014). It is known that the nitrate reductase, NarG, plays a major role in conversion of nitrite to NO (Calmels *et al.,* 1988; Ralt *et al.*, 1988; Metheringham and Cole 1997; Gilberthorpe and Poole, 2008; Vine *et al*., 2011) but some NO can be also generated from nitrite even in mutants that lack NarGHI. It has therefore been suggested that NO is generated inside the cell from nitrite by one or both of the nitrite reductases. Indeed, Corker and Poole (2003) concluded that generation of NO has been eliminated by deletion of both NrfA and NirB. Furthermore, Weiss (2006) reported that NirB might be responsible for the majority of NO production in the *E. coli* cytoplasm during anaerobic nitrate or nitrite metabolism. However, in early studies using purified NirB, production of NO or other partial reduction products from nitrite could not be detected (Coleman *et al.,* 1978). Contrary to the previous studies, Vine *et al*. (2011) found that both NirB and NrfA have a vital role in preventing the build-up of NO in the cytoplasm rather than its accumulation.

To further investigate other sources of NO in *E. coli* cytoplasm, the activity of the *hcp* promoter was assayed in a $NirB^-$ strain and a $NirB^+$ strain. The results confirmed that less NO was accumulated in the $NirB^-$ strain, which in turn caused lower activity of the *hcp* promoter compared to the $NirB^+$ strain (Figure 4.24). Incidentally, both strains are defective in nitrate and nitrite reductases and the YtfE protein but still contain the Hcp protein. This result correlated with previous findings reported by Weiss *et al.* (2006) but contradicted by Vine *et al*. (2011). In conclusion, cytoplasmic nitrite reductase, NirBDC, is another protein responsible for generation of NO in the *E. coli* cytoplasm in addition to YtfE, which has been confirmed in this study as source of NO.

5.8 Final conclusion

In conclusion, this thesis has established that OxyR does not play a significant role in regulating the response to nitrosative stress under physiological conditions. However, previous reports of possible role for OxyR can be explained by the failure of FNR to be reactivated in an *oxyR* mutant after aerobic growth to activate FNR. This work also confirmed for first time that the NsrR does not regulate transcription of the *nsrR* gene either under aerobic or anaerobic conditions. Moreover, the deletion of *nsrR* was neither beneficial nor deleterious for growth under conditions of nitrosative stress. Derepression of both *hcp* and *hmp* promoters were due to inactivation of NsrR, not FNR. Therefore, FNR does not play a key role as a physiologically relevant sensor of NO. Sources of nitrosative stress have been identified and the key results are that the YtfE is the major source of NO that has not been reported previously.

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