Stress, damage and repair during anaerobic bacterial growth

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Abstract

Bacteria are exposed to nitrosative stress generated by at least three mechanisms, all of which result in the accumulation of nitric oxide. The presence of NO results in nitrosation of iron atoms in iron-sulphur clusters. In enzymes such as aconitase and fumarase this causes inactivation. Many transcription factors containing metal cofactors, such as NsrR, sense and bind NO. NsrR is a key player in the response to nitrosative stress. When these transcription regulators bind NO, their functions are inactivated and gene expression is altered. Genes repressed by NsrR include those that code for the flavohemoglobin, Hmp, the hybrid cluster protein, Hcp, and other genes of unknown function, including ytfE. The ytfE gene encodes the di-iron protein YtfE and is reported to be involved in repair of iron-sulphur clusters damaged by nitrosative stress. We propose that Hcp and YtfE play critical roles in the response to nitrosative stress. An isogenic set of strains that lack all known (or proposed) *E. coli* NO reductases is used to investigate their roles. Strains deficient in Hcp show more sensitivity to NO under anaerobic conditions. Both *hcp* and another gene of unknown function, *ydbC*, are ruled out as encoding the unidentified NO reductase. The kinetics of the rate of NO reduction by this unidentified NO reductase is used to estimate a value for the $K_M^{app.}$ suggesting it has a very high affinity for NO. The toxic product produced by YtfE is shown to be NO. NsrR appears to be inactivated in an Hcp mutant even in the absence of nitrosative stress. The combined data suggest that there are multiple responses to nitrosative stress in *E. coli.*

Dedication

To my priority,

My gorgeous little girl

Imogen.

Acknowledgements

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Adh	Alcohol dehydrogenase
ADP	Adenosine diphosphate
Amp	Ampicillin
Arc	Arginine catabolism
Asp	Aspartate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Cat	Chloramphenicol acetyl transferase
Dms	Dimethyl sulfoxide reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
FAD	Flavin adenine dinucleotide
FNR	Regulator of fumarate and nitrite reduction
Frd	Fumarate reductase
FRT	Flp recombinase target
Fur	Ferric uptake regulation
GSNO	S-Nitrosoglutathione
Hcp	Hybrid cluster protein
Hcr	NADH oxidoreductase acting with Hcp
His	Histidine
Hmp	Flavohemoglobin
Kan	Kanamycin
K_M	Michaelis-constant, Substrate concentration at which the reaction rate is half of V_{max}
$K_M^{app.}$	Apparent K_M as is an estimate of enzymatic parameter
LacZ	Reporter gene encoding β-galactosidase
LB	Lennox Broth
$Mo[MGD]_2$	Molybdenum *bis*-molybdopterin guanine dinucleotide cofactor
MS	Minimal salts
NA	Nutrient agar

NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
Nap	Periplasmic nitrate reductase
Nar	Nitrate reductase
Nir	Nitrite reductase
NO	Nitric oxide
Nor	Nitric oxide reductase
NOSW	Nitric oxide saturated water
Nrf	Formate-dependent nitrite reductase
NsrR	Nitric oxide sensitive transcriptional regulator
Ogt	O^6-methylguanine-DNA-methyltransferase
ONPG	Orthonitrophenyl-β-D-galactopyranoside
OxyR	Oxidative stress regulator
PCR	Polymerase chain reaction
PROLI	Disodium
NONOate	1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate
PTI's	imino nitroxides
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
RIC	Repair of iron centres
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RpoS	RNA polymerase, sigma S
Sox	Superoxide
Tet	Tetracycline
TFB	Transformation buffer
TMAO	Trimethylamine-N-oxide
Tor	Trimethylamine-N-oxide reductase
V_{max}	Maximum rate achieved by system
$V_{max}^{app.}$	Apparent V_{max} as estimate of enzymatic parameter
YtfE	Iron-sulphur cluster repair protein

CHAPTER 1 – INTRODUCTION

Ability of *Escherichia coli* to adapt to anaerobic growth

Anaerobic metabolism of even simple enteric bacteria is very complex. The genetic and biochemical changes as bacteria, such as *Escherichia coli*, adapt from aerobic to anaerobic growth have been studied in detail. A key regulatory signal in facultative anaerobic bacteria is the availability of oxygen. *E. coli* adapts to a low oxygen levels, mainly by transcriptional regulation of various pathways important for anaerobic growth (Unden *et al*., 1995). In the absence of oxygen, *E. coli* can use several alternative electron acceptors for anoxic cellular respiration, including nitrate, nitrite, dimethylsulfoxide (DMSO), trimethylamine-N-oxide (TMAO) and fumarate (Unden and Dunnawald, 2007). In order for the bacterium to express the appropriate respiratory pathway, it must respond correctly to the growth environment. For this it uses a network of global and system-specific transcriptional regulators.

The regulator of fumarate and nitrate reduction, FNR, is a transcription factor that responds to oxygen starvation. During aerobic growth, where oxygen levels are high, the FNR protein is inactive. Consequently, genes that would normally be up-regulated by FNR under anaerobic conditions are switched off (Spiro and Guest, 1990). Many of the genes activated anaerobically by FNR are crucial for survival of the bacteria under these conditions (Cole, 1996). Under anaerobic conditions, FNR contains a $[4Fe\text{-}4S]^{2+}$ cluster (Lazazzera *et al*, 1996), (Khoroshilova *et al*., 1997). During aerobic growth this cluster breaks down via a $[3Fe\text{-}4S]^{+}$ cluster and a $[2Fe\text{-}2S]^{2+}$ cluster to an iron-free monomer (Khoroshilova *et al*., 1997). The $[4Fe\text{-}4S]^{2+}$ cluster allows FNR to form a

functional homodimer and bind at specific DNA sites with the consensus sequence TTGAT-N_4-ATCAA (Jayaraman *et al*., 1988) where it can either activate or repress transcription of target genes. The $[2Fe\text{-}2S]^{2+}$ cluster causes FNR to exist as inactive monomers (Khoroshilova *et al*., 1997). DNA localisation of the FNR homodimer recruits RNA polymerase to the promoter in order to activate target gene expression or alternatively it may inhibit activation of promoters by blocking the interaction of activator proteins (or RNA polymerase itself) at promoter-proximal sites, in order to repress transcription of the gene.

A variety of two-component regulatory systems function alongside FNR to enable *E. coli* cells to respond to changes in their environment (Rabin and Stewart, 1993). For example, the ArcAB system also functions in response to the availability of oxygen. ArcA is a cytoplasmic response regulator protein encoded by the *arcA* gene. By contrast, the *arcB* gene encodes the membrane-bound sensor kinase protein ArcB, which has a cytoplasmic signalling domain (Iuchi and Lin, 1988; Iuchi *et al*., 1990). Under anaerobic or microaerobic conditions ArcA represses transcription of genes involved in aerobic respiration (Iuchi and Lin, 1988; Iuchi *et al*., 1992). When oxygen is absent, ArcB is autophosphorylated at His 292. A phosphorelay mechanism proceeds whereby the phosphate group is transferred to Asp 576 of ArcB then to Asp 54 of ArcA (Iuchi et al., 1993). This phosphorylated form of ArcA can multimerise and bind at specific promoters to activate or repress transcription of the gene (Jeon *et al*., 2001). More recently, the ArcAB system has been suggested to be involved in bacterial resistance to reactive oxygen species, ROS, as well as being a global regulator for anaerobic growth (Loui *et al*., 2009; Morales *et al*., 2012).

Two other systems in *E. coli* are NarXL and NarQP (Figure 1.1 & 1.2). Both systems respond to nitrate (Figure 1.1) and nitrite (Figure 1.2) in order to alter the expression of genes required for nitrate and nitrite respiration (Rabin & Stewart, 1993). The membrane-bound sensors are NarX and NarQ, which are histidine kinases. Their respective response regulators are NarL and NarP. NarL and NarP induce gene expression in response to low levels of nitrate, as both the NarXL and NarQP systems are extremely senstitive to nitrate (Wang *et al*., 1999). NarL and NarP also respond similarly to nitrite, but activation and repression occur at a lower level (Wang & Gunsalus, 2000). Nitrate and nitrite are sensed by the membrane-bound NarX or NarQ which undergo a conformational change and become autophosphorylated on a conserved histidine residue within their cytoplasmic domains. This phosphate group is then transferred to the cognate response regulator in the cytoplasm, NarL or NarP, on to a conserved aspartate residue found in the receiver domain (Stewart, 2003). The phosphorylated forms of NarL and NarP have higher affinities for their DNA binding sites and display enhanced regulatory activity (Stewart, 1993). Important genes with a binding site in their regulatory region are either activated or repressed (Stewart & Bledsoe, 2003; Constanidou *et al*., 2006). It is important to note the asymmetry and complexity of this cross-regulation network. Either NarX or NarQ is sufficient to regulate expression of NarL dependent genes, whereas only NarX is sufficient to regulate expression of NarP-dependent genes (Egan and Stewart, 1990; Chiang *et al*., 1992). NarX interacts preferentially with NarL, whereas NarQ interacts with both NarL and NarP similarly (Noriega et al., 2010). NarX responds to nitrate preferentially whereas NarQ responds to both nitrate and nitrite similarly (Rabin and Stewart,

1993; Wang *et al*., 1999; Noriega *et al*., 2010). Also, in the presence of nitrite, NarX functions mainly to dephosphorylate NarL that has been phosphorylated by NarQ (Figure 1.2) although some kinase activity is still seen (Rabin and Stewart, 1993). The NarXL and NarQP systems are believed to interact to fine tune responses from environmental cues, such as nitrate and nitrite (Noriega *et al*., 2010).

Reduction of nitrate and nitrite during anaerobic growth

During anaerobic growth *E. coli* reduces nitrate first to nitrite, then to ammonia. Distinct pathways exist in the cytoplasmic and the periplasmic cellular compartments for the reduction of nitrate and nitrite. The cytoplasmic pathway consists of the membrane-bound quinol/nitrate oxidoreductase complex Nar and the NADH/nitrite oxidoreductase Nir. By contrast, the periplasmic pathway consists of the nitrate reductase Nap and the nitrite reductase Nrf, both of which are quinol-dependent enzymes with soluble periplasmic catalytic subunits and the electron-donor proteins, and membrane-anchored quinol-oxidase components. For each pathway, when properly coordinated, the reduction of nitrate results in the formation of ammonium. In *E.coli,* two different forms of the membrane-bound nitrate reductase are expressed. NarGHI and NarZYV are encoded by the *narGHJI* and *narZYWV* gene clusters, respectively where *narJ* and *narW* encode system-specific chaperones required for the functional maturation of each holoenzyme (Blasco *et al*., 1992; Palmer *et al*., 1996; Blasco *et al*., 1998). Both complexes are membrane bound. NarG and NarZ are the catalytic subunits where reduction is performed, both are members of the DMSO reductase protein superfamily, which contain the active site and bind the

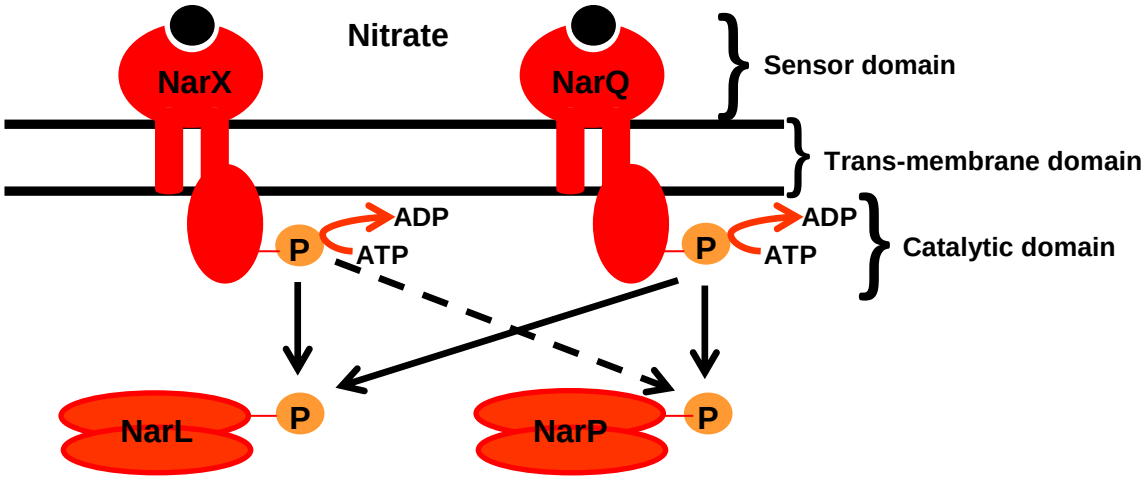


Figure 1.1 The response of the two-component regulatory systems, NarXL & NarQP, to nitrate**.**

Red arrows show autophosphorylation of the sensor and the solid/dashed black arrows show phosphotransfer to the effector, with the solid lines showing the preferential interactions.

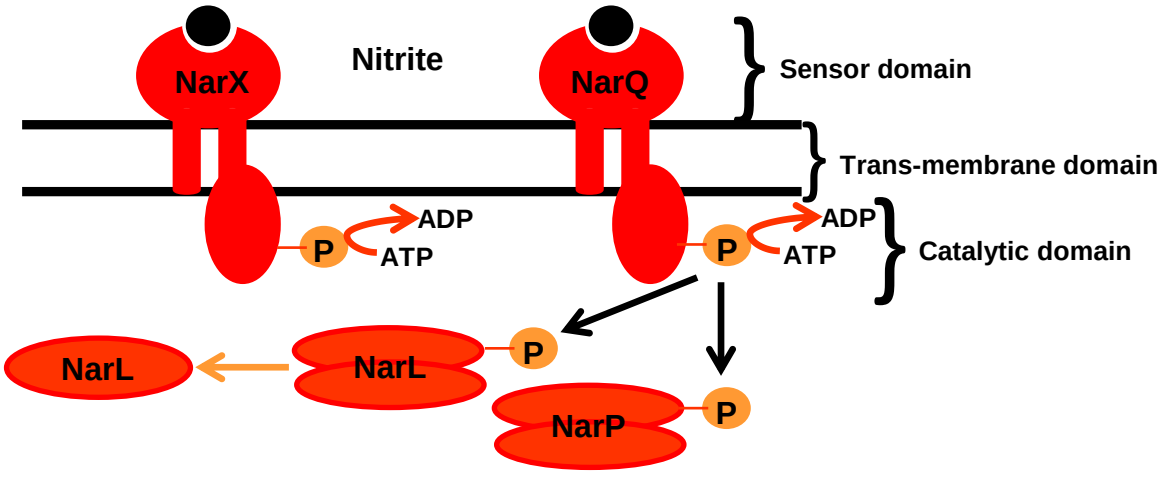


Figure 1.2 The response of the two-component regulatory systems, NarXL & NarQP, to nitrite.

Red arrows show autophosphorylation of the effector, black arrows show phosphotransfer to the effector and orange arrows show dephosphorylation.

molybdenum *bis*-molybdopterin guanine dinucleotide cofactor ($Mo[MGD]_2$) (Iobbi_Nivol and Leimkuhler, 2012). FNR activates transcription of the *narGHJI* operon under anaerobic conditions. Expression of the *narZYWV* operon is induced in the presence of nitrate by NarL. By contrast, the *narZYWV* operon is constitutively expressed at a low level during log phase growth and is regulated by the primary sigma factor for expression of stationary phase genes (RpoS) (Iobbi *et al*., 1987; Iobbi-Nivol *et al*., 1990; Spector *et al*., 1999) Accordingly, the NarZYW nitrate reductase system is known to be important during stationary phase growth of *E.coli* (Chang *et al*., 1999). Nitrite is then reduced by the cytoplasmic nitrite reductase NirBD. NirBD is an NADH-dependent protein encoded by *nirB* and *nirD* of the *nirBCD* operon, with *nirC* encoding a nitrite transport protein (Clegg *et al*., 2002). This operon is regulated in a similar manner as the *narGHJI* operon ensuring that nitrite does not build up. FNR activates the *nir* operon under anaerobic conditions and NarL and NarP activate expression in response to nitrate and nitrite.

In the periplasm, transformation of nitrate to ammonium proceeds with the reduction of nitrate to nitrite by the Nap system, for which the structural and maturation proteins are encoded by the *napDABC* and *napFGH* genes. Nitrate reduction is performed by the catalytic subunit NapA and the electron-donor NapB. NapA is a member of the DMSO reductase protein family and contains the active site and binds the $Mo[MGD]_2$ cofactor (Iobbi_Nivol and Leimkuhler, 2012). Both NapA and NapB are soluble periplasmic proteins, while other *nap* genes encode transmembrane or membrane-bound proteins that support the function of NapA. The *nap* operon is also regulated by FNR, with expression being activated under anaerobic conditions. Transcription of the *nap* operon is

increased during growth with limiting concentrations of nitrate but plenty of nitrite. The periplasmic nitrite reductase NrfA (nitrite reduction by formate) (Cole, 1996) is encoded by the *nrfA* gene. NrfA is a homodimeric enzyme that reduces the nitrite formed by NapA to ammonium (Clarke *et al*., 2008). FNR activates *nrfA* expression under anaerobic conditions and expression is further induced in the presence of low concentrations of nitrate or when nitrite is present. It is also repressed by NarXL when nitrate concentrations are high (Page *et al.*, 1990; Rabin & Stewart, 1993). Expression of *nrfA* is also regulated by the nitric oxide sensitive transcriptional repressor NsrR. In the absence of nitric oxide NsrR binds and represses transcription of the gene. However when nitric oxide is generated, NsrR binds the nitric oxide, relieving repression of transcription. Although NsrR represses expression of *nrfA*, total repression is not seen: NsrR acts more to fine tune expression.

Reduction of TMAO during anaerobic growth

Trimethylamine N-oxide (TMAO) is reduced under anaerobic conditions to trimethylamine. *E. coli* has three systems for reducing TMAO, the TorCAD system (TMAO reductase I), the DmsABC system (TMAO reductase II) and the TorYZ system (TMAO reductase III).

The *torCAD* operon encodes the inducible TMAO reductase I. The *torA* gene encodes the periplasmic catalytic terminal reductase, TorA, containing the active site and the Mo[MGD]$_2$ cofactor (Zhang *et al*., 2008; Iobbi-Nivola and Leimkuhlerb, 2012). The *torC* gene encodes the pentaheme *c*-type cytochrome subunit of the reductase, TorC. TorC is a membrane-bound protein where the globular pentaheme electron-transfer domain is anchored to the

periplasmic-face of the cytoplasmic membrane to allow the passage of electrons to TorA (Gon *et al*., 2001; Ilbert *et al*., 2004). The *torD* gene encodes a chaperone protein, TorD, that is specific for TorA. TorD binds TorA before it receives its ($Mo[MGD]_2$) cofactor and promotes maturation. TorA is then secreted into the periplasm (Chan *et al*., 2009; Ilbert *et al*., 2004). The *torCAB* operon is induced by anaerobiosis (Simon *et al*., 1994), repressed by NarL in the presence of nitrate and induced in the presence of TMAO or related compounds by the two component regulatory system TorS/TorR (Jourlin *et al*., 1996).

In this two component regulatory system, TorS is a sensory histidine kinase, encoded by *torS*. TorS responds to changes in the concentration of TMAO in the periplasm. The presence of TMAO is sensed by the N-terminal ligand binding domain of TorS, which on TMAO binding triggers autophosphorylation at its transmitter domain at a conserved histidine residue. This phosphoryl group is then transferred to a conserved aspartate residue in the receiver domain before being transferred to another distinct site comprising a histidine residue within the signalling domain of the C-terminal. The phosphorylated transmitter domain of TorS acts as the phosphodonor to TorR, and is received at the cognate receiver domain present in this DNA-binding regulator. This is a four stage phosphorelay (Jourlin *et al*., 1997). TorR, encoded by *torR*, is the response regulator component of the TorS/TorR system. It regulates expression of the *torCAD* operon according to the concentration of TMAO present by binding upstream of TorC (Simon *et al*., 1995). The two component system is regulated in different ways. Phosphorylated TorR regulates expression of the *torR* gene (Ansaldi *et al*., 2000). Immature TorC interacts with TorS and inhibits

its kinase activity (Ansaldi *et al*., 1999). It has also been suggested that TorS can dephosphorylate TorR by reverse phosphorelay when TMAO is removed (Ansaldi *et al*., 2001). Two other proteins, TorI and TorT, play a role in the TorS/TorR system. TorI works downstream of TorS phosphorylation and requires TorR to negatively regulate expression of the *torCAD operon*, but not by dephosphorylating TorR or by stopping it binding to the DNA. It has been suggested that it may prevent recruitment of RNA polymerase (Ansaldi *et al*., 2004). TorT is also involved in the regulation of the *torCAD* operon by association with the TorS/TorR two component regulatory system. TorT binds TMAO promoting a conformational change cascade from TorT to TorS causing activation of TorS (Baraquet *et al*., 2006).

The *dmsABC* operon encodes a dimethyl sulfoxide (DMSO) reductase that also reduces TMAO (TMAO reductase II). The subunit containing the active site and the Mo[MGD]$_2$ cofactor, DmsA, is encoded by *dmsA* (Weiner *et al*., 1988). Subunit DmsB contains a [4Fe-4S] cluster and is responsible for electron transfer. DmsC stabilizes and anchors DmsA and DmsB to the cytoplasmic side of the plasma membrane (Sambasivarao *et al*., 1990). The *dmsABC* operon is induced by FNR in the absence of oxygen and expression is inhibited by NarL in the presence of nitrate (Cotter and Gunsalas, 1989; Bearson *et al*., 2002). Both of these effects have been shown to require molybdenum (Cotter and Gunsalas, 1989; McNicholas *et al*., 1998).

The third TMAO reductase system found in *E. coli* is encoded by the *torYZ* operon (TMAO reductase III). TorZ is the catalytic subunit of this periplasmic TMAO reductase with 42% sequence identity to TorA. TorY is a pentahemic *c*-type cytochrome, homologous to TorC. TorY is anchored to the inner

membrane. This system also has broad substrate specificity and is able to reduce N- and S-oxide compounds (Gon *et al*., 2000). Unlike the other two systems, TorYZ is expressed at low levels and is not induced by TMAO or DMSO, it is constitutively expressed (Gon *et al*., 2000).

Reduction of Fumarate during anaerobic growth

When fumarate is used as a terminal electron acceptor fumarate reductase, encoded by *frdABCD*, catalyses the reduction of fumarate to succinate (Spencer and Guest, 1973). FrdA and FrdB are the catalytic subunits of the reductase, FrdA contains a flavin adenine dinucleotide (FAD) cofactor, while FrdB contains three different iron-sulphur clusters, a [4Fe-4S] cluster, a [3Fe-4S] cluster and a [2Fe-2S] cluster (Iverson et al., 1999). FrdC and FrdD anchor the enzyme to the cytoplasmic side of the inner membrane and bind menaquinol, the electron donor (Iverson *et al*., 1999). Transcription of the fumarate reductase operon is regulated by the levels of oxygen, nitrate and fumarate (Jones and Gunsalas, 1985). Expression of FrdABCD is activated under anaerobic conditions by FNR, and is also activated in the presence of fumarate. It is repressed in the presence of nitrate by the NarXL and NarQP two component regulatory systems (Jones and Gunsalas, 1987).

Exposure to reactive nitrogen species

Bacteria need to be able to defend themselves against reactive nitrogen species (RNS) from four sources: products of their own metabolism, especially during nitrate and nitrite reduction; products of other bacteria sharing their environment, again from nitrate and nitrite reduction; NO generated as part of a mammalian host defence mechanism; or as products of non-specific chemical

reactions (Filenko *et al.*, 2007). NO is produced both aerobically and anaerobically. NO is produced aerobically by macrophages as part of the mammalian host defence mechanism. More NO is produced anaerobically as nitrate reduction is regulated by FNR, which is only active in the absence of oxygen. Nitrate reduction occurs mainly anaerobically. RNS show varying degrees of toxicity. Nitrate, for example, is virtually harmless unless metabolized to nitrite, but nitric oxide is significantly damaging to bacteria.

It has been predicted that NO is generated as a side product of nitrite reduction to ammonia by either NirBD or NrfA (Corker & Poole, 2003; Weiss, 2006). Alternatively, the cytoplasmic nitrate reductase, NarG, has also been implicated in production of NO upon interaction with nitrite in enteric bacteria (Calmels *et al.*, 1988; Ralt *et al*., 1988; Metheringham *et al*., 1994). More recently NarG was confirmed to be the major source of NO (Gilberthorpe & Poole, 2008; Vine *et al.*, 2011; Rowley *et al*., 2012; Maia and Moura, 2014). In the presence of nitrate, production of NO by NarG from nitrite is inhibited.

Effects of nitrosative stress in anaerobic environment

The lifetime of NO is increased in an anaerobic compared to an aerobic environment. In an anaerobic environment there is increased damage as NO is around longer before being reduced. Complex and effective systems are required for the detoxification of NO under anaerobic conditions. NO causes altered gene expression throughout the *E. coli* genome, including genes encoding detoxification enzymes, genes whose products are required for the assembly of iron-sulphur clusters, DNA repair enzymes and also regulators of the stress response (Justino *et al.*, 2005; Filenko *et al*., 2007).

Proteins containing iron-sulphur clusters are key targets of nitrosative stress. The presence of NO results in nitrosation of the iron atoms in the iron-sulphur clusters. Nitrosation of iron-sulphur clusters in enzymes such as aconitase or fumarase causes inactivation of the enzyme (Justino *et al.*, 2007).

Detoxification of NO by *E. coli*

Three different proteins in *E.coli* detoxify NO. These are the flavohemoglobin, Hmp, the flavorubredoxin, NorV, and the cytochrome *c* nitrite reductase, NrfA (Bodenmiller & Spiro, 2006). The first to be identified and the best understood protein for removal of NO is Hmp (Gardner and Gardner, 1998; Hausladen *et al.*, 1998). Hmp protects cells against nitrosative stress (Corker & Poole, 2003). It is inducible under both aerobic and anaerobic conditions by nitrate but more so by nitrite and NO. In the presence of oxygen, Hmp oxidizes NO rapidly to nitrate. In the absence of oxygen, Hmp may detoxify NO by reducing it to N_2O (Figure 1.3). The *hmpA* promoter is repressed by FNR in an anaerobic environment (Cruz-Ramos *et al*., 2002). When nitrosative stress is extreme, signalled by nitrate or especially nitrite or NO, the iron-sulphur cluster of FNR undergoes nitrosation. FNR becomes inactive and the *hmpA* promoter is upregulated (Corker & Poole, 2003). Another transcriptional regulator regulates the *hmpA* promoter in response to nitrosative stress. This is the NsrR protein, a nitric oxide-sensitive transcription regulator that represses *hmpA,* especially under anaerobic conditions until nitrosative stress relieves this repression (Bodenmiller & Spiro, 2006).

NorVW is an NO reductase (Figure 1.3). Other NO detoxification systems, such as Hmp, are regulated by FNR. FNR is only active when no oxygen is present,

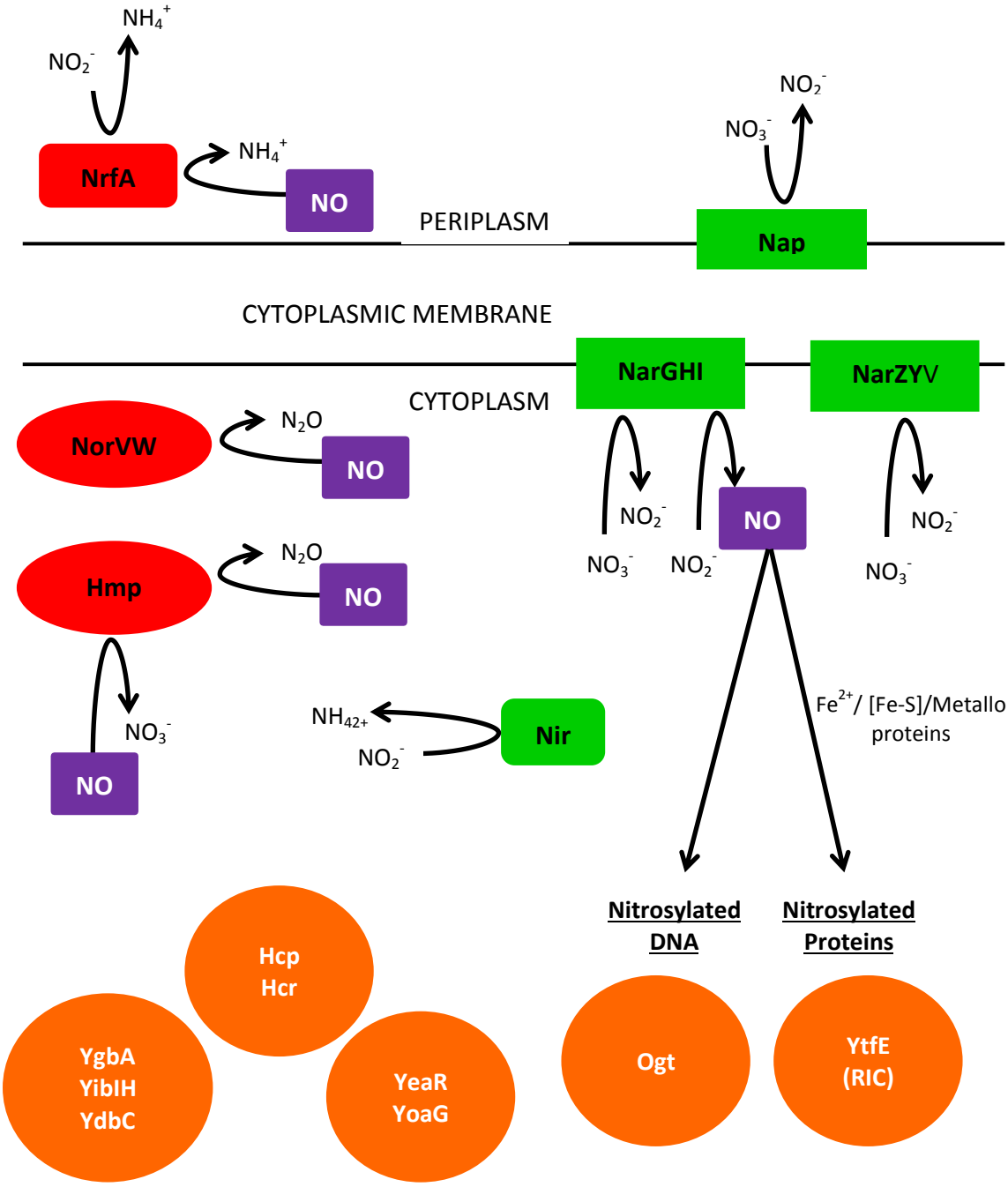


Figure 1.3 The proteins involved in NO generation and removal, and those involved in repair of damage by NO.

Nitrate and nitrite reductases are shown in green. Enzymes that reduce NO are shown in red and proteins known or suspected to be involved in repair of damage are shown in orange.

i.e. when an alternative terminal electron acceptor is being used. When nitrate or nitrite is being used NO is produced, so expression of the NO reductase is regulated by FNR. NorVW, however, is not FNR dependent (Bodenmiller & Spiro, 2006). NorVW is activated by NorR. NorR is a σ^{54}-dependent transcription factor, a DNA-binding protein and an NO sensor (Gardner *et al*., 2003; Hutchings *et al*., 2002). σ^{54}, unlike other sigma factors, is incapable of opening double-stranded DNA, σ^{54} proteins require ATPase activity of transcriptional activators, like NorR, to activate transcription of the target gene by changing the conformation of the DNA (Schumacher *et al*., 2006; Wigneshweraraj *et al*., 2008; Shingler *et al*., 2011). Nitrosylation of the di-iron centre of NorR occurs even when the cytoplasmic concentration of NO is low. NorR now promotes transcription of the *norVW* operon. NorV is a reductase that reduces NO to nitrous oxide and NorW is a redox protein that reduces NorV.

Nrf also functions to reduce NO to ammonia under anaerobic conditions (Clarke *et al*., 2008: van Wonderen *et al*., 2008). Transcription of the *nrf* operon is activated in response to low levels of nitrate and repressed when nitrate concentrations are high. Strains containing all of the above-mentioned NO reductases and a *nirBD* mutation singularly and in combinations still showed NO reductase activity, suggesting the presence of another unknown mechanism for NO reduction, possibly an unidentified NO reductase (Vine & Cole, 2011).

Sensors of NO

Many transcription regulators containing metal cofactors sense and bind NO. Some, such as NsrR and NorR, are believed to sense NO as their primary function. Others, such as FNR and Ferric uptake regulator (Fur) are less

sensitive to NO and sense another, primary, signal. When these transcription regulators bind NO, their functions are inactivated and gene expression is altered (Spiro, 2007).

SoxR, the superoxide response protein, is activated in the presence of superoxide (Pomposiello and Demple, 2001). Activated SoxR induces transcription of the *soxS* gene, which encodes the response regulator SoxS. SoxS activates expression of many genes involved in the response to oxidative stress (Touati *et al*., 2000). While its primary function is to respond to superoxide, SoxR has also been shown to respond to NO to produce the same response. Other activated genes, such as genes involved in repair of iron- sulphur clusters, may be of more use in the presence of NO (Giro *et al*., 2006).

OxyR, the main regulator in response to oxidative stress, in particular hydrogen peroxide, has also been suggested to be regulated by nitrosative stress, although not by NO itself (Hausladen *et al*., 1996; Kim *et al*., 2002; Justino *et al*., 2005). In a recent study by Seth *et al*. (2012), OxyR was identified as playing an important role under anaerobic condition in the presence of nitrosative stress. Although classically aerobic, S-nitrosylation was proposed to occur anaerobically in the presence of nitrate, and be controlled by OxyR, as deletion of OxyR resulted in a large increase in the amount of S-nitrosylated proteins. An OxyR mutant also showed inhibited growth anaerobically in the presence of nitrate, increased S-nitrosylation was sufficient to impair growth. OxyR, and possibly other genes in its regulon, may protect against nitrosative stress by increasing protein denitrosylation. Also, an OxyR mutant showed transcriptional change of genes involved in metabolizing NO, such as *hmp* and

norVW and other genes known to be important in the nitrosative stress resonse, such as *hcp*.

The ferric uptake regulator (Fur) is a transcription factor that is primarily regulated by iron. When iron is bound by Fur, repression occurs. It regulates genes involved in iron acquisition and iron-sulphur cluster repair proteins (Andrews *et al*., 2003). Although an iron sensor, Fur has been shown to respond to NO under iron-limited conditions (D'Autr´eaux *et al*., 2002). In the presence of NO, Fur repressed genes become induced due to Fur being unable to bind the DNA.

In the presence of NO, FNR has been shown to be inactivated, altering expression of genes involved in the nitrosative stress response, such as Hmp (Poole *et al*., 1996).

Although the above regulators respond to NO as a secondary role, there are regulators that sense nitric oxide as the primary function. These include NorR and NsrR.

As previously mentioned, NorR regulates expression of the NorVW operon, encoding the flavoreubredoxin NO reductase, NorV, and its redox protein, NorW. Under conditions of low NO, NorR becomes nitrosylated and binds the promoter region of the NorVW operon as a trimer and promotes expression of the genes.

NsrR, the nitrite sensitive repressor protein, is another specific sensor of NO. NsrR regulates transcription of many genes involved in the response to nitrosative stress (Bodenmiller and Spiro, 2006). NsrR contains an NO-sensitive

[2Fe-2S] cluster, which is required for NsrR to be able to bind and repress transcription (Tucker *et al*., 2008; Tucker *et al*., 2010). In the presence of NO, this cluster becomes nitrosylated, NsrR can no longer bind the DNA and expression of the gene is derepressed. The genes shown to be derepressed are important in the nitrosative stress response, these include *hmp*, *hcp-hcr* and *ytfE* (Bodenmiller and Spiro, 2006; Filenko *et al*., 2007). It has also been suggested that NsrR is inactivated when the availability of iron is limited, either directly or indirectly (Bodenmiller and Spiro, 2006).

Repair of damage caused by NO by YtfE

As proteins that contain iron-sulphur clusters are one of the main cellular targets for nitrosative damage, the focus on repair of damage due to nitrosative stress often becomes focus on repair of iron-sulphur proteins. The *ytfE* gene, encoding the di-iron protein YtfE, is strongly induced by NO, nitrate and nitrite in anaerobic cultures. This activation by nitrate and nitrite is not due to the NarXLQP system (Bodenmiller and Spiro, 2006). Nitrate and nitrite are reduced to NO. NO relieves repression by NsrR. NsrR represses expression by directly binding the promoter in the absence of nitric oxide. FNR represses expression indirectly (Vine, PhD Thesis, 2011). YtfE was revealed to be involved in anaerobic NO protection in *E. coli.* It has been shown that a YtfE mutant shows inhibited growth under anaerobic conditions with fumarate or nitrite, both of which involve enzymes with iron-sulphur clusters. Iron-sulphur cluster enzymes, aconitase and fumarase were less active in a YtfE mutant, and a YtfE mutant also showed increased sensitivity to hydrogen peroxide and no ability to repair fumarase activity inactivated by oxidative stress (Vine *et al*., 2010). A *ytfE* mutant grew poorly under anaerobic conditions and showed increased

sensitivity to iron starvation (Justino et al., 2007). YtfE has repeatedly and conclusively been shown to be necessary for the repair of iron-sulphur centres damaged by stress (Justino *et al.*, 2005; Justino *et al*., 2006; Justino *et al.*, 2007; Overton *et al.*, 2008; Vine *et al.*, 2010). Also, a YtfE orthologue from *gonococcus*, DnrN, is critical for protection during both oxidative and nitrosative stress (Overton *et al*., 2008).

The Hybrid Cluster Protein, Hcp

A microarray study by Constantinidou *et al.* (2006) identified several other genes of unknown function that may play a role in resistance to nitrosative stress (Table 1). The most studied is the hybrid cluster protein, encoded by the *hcp* gene (Flatley *et al*., 2005; Almeida *et al*., 2006; Filenko *et al*., 2007). The *hcp* gene occurs in an operon with the *hcr* gene, which encodes an NADH oxidoreductase, Hcr, that reduces Hcp. The structure of the Hcp protein is so far unique as it contains both a [4Fe-4S] cluster and a hybrid [4Fe-2S-2O] cluster (van der Berg *et al*., 2000). Hcp has been widely studied, yet its role still remains elusive. Hcp is said to be a hydroxylamine reductase that protects bacteria from hydroxylamine toxicity during reduction of nitrite to ammonia. However, this occurs at an optimum pH 9.0 (Wolfe *et al*., 2002). Also, the Michaelis-constant (K_M value) of Hcp from *E. coli* for hydroxylamine is very high, (38.9 +/- 3.53 mM at pH 7.5 and 2.5 +/- 0.36 mM at pH 9.0), several orders of magnitude more that the growth inhibitory concentration (Wolfe *et al*., 2002; Filenko *et al*., 2007). Hcp was unable to protect against hydroxylamine stress in *Wolinella succinogenes* (Kern et al., 2011) and the presence of hyroxlamine in the growth medium had no effect on the level of expression of Hcp (Squire, PhD Thesis, 2009) As Hcp's ability to reduce hydroxylamine is not physiologically

Table 1: Genes of unknown function that may play a role in resistance to nitrosative stress

Protein	Function	Transcription Regulation-Activation(+), Repression(-)	Reference
Hcp	*Unknown, debated	FNR (+) NsrR (-)	Wolfe *et al.*, 2002; Constantinidou *et al.* 2006
YgbA	Unknown	NsrR (-)	Filenko *et al*., 2007
YeaR-YoaG	Unknown	NarXL (+)	Constantinidou *et al*., 2006
YibIH	Unknown	FNR (+) NarXL (+)	Overton *et al*., 2006
YdbC	Putative oxidoreductase	NsrR (+)	Filenko *et al*., 2007
**AdhC	S-glutathionehydroxamate reduction	FNR (+) NarL (-)	Leonardo *et al.*, 1993; Liu *et al*., 2001
YccM	Unknown	Predicted 4Fe-4S membrane protein	Filenko *et al*., 2007

* Documented as hydroxylamine reductase, see text.

** AdhC is also known as AdhE. It was first identified as an ethanol dehydrogenase, but also has other functions

relevant, alternative roles are being considered (Vine & Cole, 2011). Hcp has been implicated in the defence against oxidative stress (Almeida *et al*., 2006). However, conditions were not appropriate for anaerobic respiration and the V_{max} is too low to confirm that Hcp is a peroxidase. Hcp has been implicated in the response to nitrosative stress (Justino *et al*., 2005; Filenko *et al*., 2007). It has been proposed as the unidentified NO reductase and also suggested to be involved in the repair pathway protecting the cytoplasm of the bacteria from nitrosative stress (Vine & Cole, 2011). Hcp is most highly expressed under anaerobic conditions in the presence of nitrate or nitrite (van den Burg et al., 2000). Hcp has been shown to be upregulated anaerobically, but not aerobically, by NO (Pullan *et al*., 2007) and both anaerobically and aerobically by S-Nitrosoglutathione (GSNO) (Flatley *et al*., 2005). In a more recent study by Seth *et al*. (2012), Hcp was again implicated in protection against nitrosative stress and was again suggested to be relevant under both anaerobic and aerobic conditions.

The *hcp* promoter is regulated similarly to other genes involved in nitrosative stress, including *nirB* and *narG*, which encode proteins that generate nitric oxide. Expression is induced during anaerobic growth in the presence of nitrite (Filenko *et al*., 2007). However, this regulation is not due to NarXLQP as binding sites at the *hcp* promoter showed only a minor role in regulation (Chismon *et al*., 2010). Hcp is also activated by FNR under anaerobic conditions by direct binding of the activator (Chismon *et al*., 2010). Hcp expression is repressed by the nitrite-sensitive repressor, NsrR, again directly by binding to the promoter (Chismon *et al*., 2010). This repression is relieved in the presence of nitric oxide (Filenko *et al*., 2007).

The structure of the Hcp protein consists of three domains. Domain 1 contains two three-helix bundles joined by a nine amino acid linker. This linker loops around the [4Fe-4S] cluster (Cooper *et al*., 2000). Domains 2 and 3 contain a beta sheet that is surrounded by alpha helices, giving a Rossman fold structure in both domains (Aragão *et al.,* 2008).

The Hcp protein is found in other organisms capable of anaerobic respiration, such as *Morganella morganii* and in other strictly anaerobic organisms, such as *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans*. The highly conserved cysteine residues responsible for coordinating the hybrid cluster at the centre of the protein are always conserved as are the cysteine residues that coordinate the conventional cluster (van den Burg *et al*., 2000). Hcp is not present in *Bacillus subtilis*, nor in some bacteria with small genomes, such as *Mycobacterium sp.* and *Haemophilus influenzae*.

Assay for detecting accumulation of NO in *E. coli* cytoplasm

A new assay has been developed for the detection of NO accumulation in the bacterial cytoplasm, based on the NsrR transcription factor. NsrR represses *hcp* and *hmp* expression, and is sensitive to low concentrations of NO in the cytoplasm (Bodenmiller & Spiro, 2006). In the absence of NO the *hcp* promoter is repressed by NsrR. When NO accumulates, repression is relieved and expression of *hcp* is increased. A reporter system was developed based upon a plasmid that contained an *hcp::lacZ* fusion. β-galactosidase activity was therefore dependent on relief of NsrR repression of the *hcp* promoter in response to NO (Filenko *et al*., 2007; Chismon *et al*., 2010; Vine *et al*., 2011).

Aims of this project

Previous experiments by Vine (PhD thesis, 2011) investigated the effect of various NO reductase mutations on NO accumulation in *E.coli*. These NO reductase mutants were grown anaerobically under various conditions and the rate of NO reduction for the same mutants was measured. Deletion of all characterized NO reductases in *E. coli* (*nirBD, norVW, nrf* and *hmp*) produced a mutant strain that was still able to reduce NO at a rate similar to the parent. One or more previously unreported NO reductases must therefore exist. Identification of a potentially uncharacterized and additional system for NO reduction is a central objective of this project. Investigation into the kinetics of the rate of NO reduction is also an aim of this project. A potential gene of interest is *ydbC*, since transcription of this gene was shown to be upregulated by the NO-responsive regulator NsrR (Filenko *et al*., 2007).

Many variations of deletions of these NO reductases, *nirBD, norVW, nrf and hmp* in combination with mutations in *hcp* and *ytfE* were made during generation of other strains (Table 2.1). Investigating the growth phenotypes and the effect of regulation by NsrR of these strains is also an aim of this project. Another aim of this project is to discover the function of Hcp and further investigate the role of YtfE in repair of damage by nitrosative stress.

As YtfE has been shown to play a role in the repair of Fe-S clusters and Hcp has been suggested to play a role in the response to nitrosative stress, two proteins whose Fe-S cluster is damaged by nitrosative stress will be investigated. These are aconitase and fumarase. Their activity will be assayed and any effects due to mutations will be identified.

CHAPTER 2 - MATERIALS AND METHODS

Media

The required amount of solid for either liquid or solid media was dissolved in distilled water and autoclaved to sterilise at 121°C and 1 atmosphere pressure for 15 minutes. Alternatively liquids were sterilised by filter sterilisation using a 0.2 µm pore filter. Lennox broth (LB) consisted of 20 g L^{-1} Tryptone, 10 g L^{-1} yeast extract and 10 g L^{-1} NaCl. Minimal salts (MS) medium consisted of 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 µM ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$), 1 µM sodium selenite (Na_2SeO_3) and 1 ml L^{-1} *E. coli* sulphur free salts. *E. coli* sulphur-free salts contained 82 g L^{-1} $MgCl_2.7H_2O$, 10 g L^{-1} $MnCl_2.4H_2O$, 4 g L^{-1} $FeCl_2.6H_2O$, 1 g L^{-1} $CaCl_2.6H_2O$ and 20 ml L^{-1} concentrated HCl. Nutrient agar (NA) was used at 28 g L^{-1} and was supplied by Oxoid, Hampshire, UK.

Bacterial strains

The strains used in this study are listed in Table 2.1. The deletions were transferred to various recipients by bacteriophage P1 transduction, or were made by the Datsenko and Wanner method of chromosomal gene inactivation. The mutant that is missing the three known NO reductases (*nir*, *nrf* and *norVW*) will be referred to throughout this report as the triple mutant, any variation of this mutant in the *hmp*, *hcp* or *ytfE* genes known to be involved in nitrosative stress will be stated.

Antibiotics

The required amount of antibiotic was dissolved in the appropriate solvent, the solution was sterilised by filter sterilisation and stored at 4 °C or -20 °C (Table 2.2).

Buffers

All buffers and solutions used are described in Table 2.3.

Preparation of bacteria for long term storage

A single colony of the required strain was used to inoculate 1 ml of LB. This culture was aerated at 37 °C until the optical density (OD) at 650nm had reached approximately 0.7. In a sterile screw-top 2 ml tube, 0.7 ml of the culture and 0.3 ml of 50% v/v sterile glycerol were mixed. The tube was snap frozen by submerging for a few seconds in liquid nitrogen, and then stored at -80°C.

Plasmids

Plasmids were isolated by small-scale DNA purification using a QIAprep Spin Miniprep kit or a Bioline Miniprep kit. Plasmids used in this work are shown in Table 2.4.

Preparation of competent *E. coli* cells

A single colony was used to inoculate 1 ml LB supplemented with relevant antibiotic. The culture was aerated at 37 °C overnight. A 200 µl sample of the overnight culture was used to inoculate 20 ml LB containing the relevant antibiotics. The culture was aerated at 37 °C for around 2 hours until the optical density at 650 nm had reached 0.5. The cells were centrifuged at 4000 *g* for 3

Table 2.1: *E. coli* K-12 strains used in this study

Strain	Description	Source
JCB 4999	RK4353 *Δhcp::cat*	Filenko, PhD Thesis, 2005
RK4353	*lacU169 araD139 rpsL gyrA non*	Stewart and McGregor, 1982
RK4353 *ΔytfE*	RK4353 *ΔytfE:: cat*	Vine, PhD Thesis, 2011
JCB5228	RK4353 *ΔytfE::cat*^{S} *Δhcp::cat*	Vine, PhD Thesis, 2011
JCB5222	RK4353 *ΔnsrR:: kan*	Vine, PhD Thesis, 2011
JCB5232	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::cat*	Vine, PhD Thesis, 2011
JCB5210	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::catS Δhmp::kan*	Vine, PhD Thesis, 2011
JCB5242	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::catS Δhcp::cat*	Vine, PhD Thesis, 2011
JCB5250	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::catS Δhmp::kan Δhcp::cat*	Vine, PhD Thesis, 2011
JCB5260	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::catS Δhmp::kanS Δhcp::catS ΔytfE::cat*	Vine, PhD Thesis, 2011
JCB5257	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::catS Δhmp::kan ΔytfE::cat*	Vine, PhD Thesis, 2011
JCB5270	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::catS Δhmp::kan Δhcp::cat ΔnarZ::Ω ΔnarGHJI*	Vine, PhD Thesis, 2011
JCB5280	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::catS Δhmp::kanS Δhcp::catS ΔytfE::cat ΔnarZ::Ω ΔnarGHJI*	Vine, PhD Thesis, 2011
RK4353 *ΔydbC*	RK4353 *ΔydbC ::kan*	Keio, Baba *et al*., 2006
JCB5210 *ΔydbC*	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::catS Δhmp::kanS ΔydbC ::kan*	Basema Balasiny University of Birmingham, 2012
JCB5251	RK4353 *ΔnirBDC::kanS ΔnrfAB::catS ΔnorV::catS Δhmp::kan Δfnr::cat*	Vine, PhD Thesis, 2011

Table 2.2: Antibiotics

Antibiotic	Stock concentration (mg ml^{-1})	Working concentration (µg ml^{-1})	Solvent	Storage (ºC)
Carbenicillin	100	100	Water	4
Kanamycin	100	25	Water	4
Chloramphenicol	50	15 or 25	Methanol	-20
Tetracycline	10	35	50 % Ethanol	-20
Rifampicin	50	25	Methanol/ DMSO	4

Table 2.3: Buffers and solutions

Name	Composition
General buffers	
Phosphate buffer (50 mM, pH 7.4).	7.26 g L^{-1} K_2HPO_4 and 1.13 g L^{-1} KH_2PO_4
Buffers for making competent *E. coli* cells.	
TFB 1.	100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$ and 15% (v/v) glycerol.
Buffers for β-galactosidase assays	
Z buffer.	KCl (0.75 gL^{-1}), $MgSO_4.7H_2O$ (0.25 gL^{-1}), Na_2HPO_4 (8.55 gL^{-1}), $NaH_2PO_4.2H_2O$ (4.87 gL^{-1}) and β-mercaptoethanol (2.7 mL per L).
1xA, Potassium phosphate buffer (0.1M, pH 7.0).	K_2HPO_4 (11.6 gL^{-1}) and KH_2PO_4 (4.54 gL^{-1}).
Sodium deoxycholate (1 %).	Sodium deoxycholate (1 g) in distilled water (100 mL).
Ortho-nitrophenyl β–D-galactopyranoside (ONPG, 13 mM).	ONPG (0.0392 g) in 1 x A (10 mL).
Buffers for electrode assays	
Assay buffer.	Phosphate buffer (50 mM, pH 7.4), supplemented with 50 µM EDTA, and 0.4% v/v glycerol.
Electrolyte.	17.5 g KCl in 100 mL distilled water.
Buffers for aconitase assays	
Assay buffer	Tris-HCl (10 mM pH 7.4) supplemented with 0.6 mM $MnCl_2$.
Sodium citrate (1M)	Sodium citrate (103.23g) in 400 ml of assay buffer.
Solutions for fumarase assays	
Sodium glutamate (0.1 M)	Sodium glutamate (0.169g) in 10 ml phosphate buffer.
Sodium fumarate (1 M)	Sodium fumarate (1.60g) in 10 ml H_2O.
Reagents for Folin assays	
Folin A solution	2% Na_2CO_3 in 0.1 M NaOH
Phenol reagent	10 ml phenol reagent with 7 ml distilled water

Table 2.4: Plasmids

Plasmid	Description	Reference or source
pNF383	The *hcp* regulatory region cloned into pPRW50 to give an *hcp:lacZ* fusion. Tet^R.	Filenko *et al.,* 2007
pSP01	*phmp* cloned into the *Eco*R1/*Hin*dIII restriction sites of pRW50. Tet^R.	Claire Vine, PhD Thesis, University of Birmingham, 2011
pCV01	*pytfE* cloned into the *Eco*R1/*Hin*dIII restriction sites of pRW50. Tet^R.	Claire Vine, PhD Thesis, University of Birmingham, 2011
pGIT	205 bp *ytfE* promoter fragment in pSTBlue-1	Bodenmiller & Spiro, 2006
pACYC184 Hcp-Hcr	A functional Hcp-Hcr operon cloned into pACYC184. Amp^R.	Claire Vine, PhD Thesis, University of Birmingham, 2011
pCP20	The Flp recombinase gene (exo) under the control of a heat sensitive promoter (active at 30ºC). $Chlor^R$ and $Carb^R$.	Datsenko and Wanner, 2000

minutes, the supernatant was discarded and the cells were resuspended in TFB1 buffer (Table 2.3) and incubated on ice for 90 minutes. The cells were centrifuged at 4000 g for 3 minutes, the supernatant was discarded and the cells were resuspended in 0.1 M $CaCl_2$ supplemented with 15 % (v/v) glycerol. Aliquots of 100 µl were snap-frozen by submerging in liquid nitrogen for a few seconds and then were stored at -80 °C.

Transformation of *E. coli* cells with plasmid DNA

Plasmid DNA, 1 – 5 µl was added to 50 µl competent cells, and the mixture was incubated on ice for 40 minutes. The cells were heat-shocked at 42 °C for 2 minutes then incubated on ice for 5 minutes. The cells were resuspended in 1 ml LB and transferred to a sterile test tube. The culture was aerated at 37 °C for 1-2 hours to allow bacteria to recover and express the antibiotic resistance proteins. A 100 µl sample was plated onto selective agar containing the required antibiotic and incubated at 37 °C overnight.

Removal of antibiotic resistance cassette using FRT sites

A 1 µl aliquot of pCP20 plasmid DNA was mixed with 50 µl of competent cells of the resistant strain, chloramphenicol or kanamycin. The mixture was incubated on ice for 40 minutes then heat shocked at 42 °C for 2 minutes before being incubated on ice again for 5 minutes. The mixture was resuspended in 1 ml of LB and transferred to a sterile test tube, which was aerated for 1 hour at 30 °C. An aliquot of 100 µl was plated onto NA supplemented with 100 mg ml^{-1} carbenicillin which was then incubated at 30 °C overnight. Individual colonies were purified on NA without any antibiotic and incubated at 42 °C overnight. Individual colonies were ‘patched’ onto NA without antibiotic, NA supplemented

with carbenicillin and NA supplemented with kanamycin or chloramphenicol. Colonies that grew on NA but were sensitive to carbenicillin or kanamycin/chloramphenicol were checked by colony PCR to have lost the antibiotic cassette. To do this, primers that flank the gene of interest were used.

Growth experiments with nitrate or nitrite

Cells were grown anaerobically in minimal medium supplemented with 5 % (v/v) LB, 0.4 % (v/v) glycerol of glucose, 20 mM TMAO and 20 mM sodium fumarate in 50 ml or 100 ml conical flasks. All cultures were started with 2 % (v/v) (unless otherwise stated) aerobic innocula grown in LB overnight with aeration at 37 °C. The optical density at 650 nm was monitored until it had reached approximately 0.2, then 5 mM nitrate or either 0.5 or 2.5 mM nitrite was added to one culture while the other was left as an unsupplemented control.

Growth experiments with 2,2-2,2-dipyridyl and Fe

Some cultures, grown as above, were supplemented with 100 μM or 300 μM 2,2-2,2-dipyridyl or 100 μM, 200 μM or 500 μM $FeSO_4$ as well as with nitrate or nitrite, others were supplemented with $FeSO_4$ only, without nitrosative stress. The optical density at 650 nm was monitored for several hours following these additions.

Preparation of nitric oxide saturated water (NOSW)

A volume of 5 ml distilled water at pH3 was pipetted into a glass bijoux bottle, the bottle was then closed with a turnover rubber stopper (Fisher Scientific, Leicestershire, UK). Silicone grease was applied to the rubber septum then two needles were inserted into the bottle. One was pushed to the bottom of the

liquid and a shorter one into the headspace above the water. First oxygen-free dinitrogen gas, then nitric oxide was passed through silicone tubing, into a flask of 3 M NaOH then into a flask of distilled water before passing to the bijoux bottles containing the pH3 water. The water was bubbled first with nitrogen for 30 minutes, then with nitric oxide for 30 minutes to produce water that was saturated with nitric oxide at approximately 2 mM. The needles were removed and the bottles were sealed with Parafilm to prevent any oxygen leaking into the vessel. When required, NOSW was removed from the bottles using a Hamilton syringe.

Growth experiments with nitric oxide

Cells were grown anaerobically in minimal medium supplemented with 5 % (v/v) LB, 0.4 % (v/v) glycerol, 20 mM TMAO and 20 mM sodium fumarate in 50 ml or 100 ml conical flasks. All cultures were started with 2% (v/v) aerobic inoculum (unless otherwise stated) grown in LB overnight with aeration at 37 °C. Anaerobic cultures were incubated statically at 37 °C. The optical density at 650 nm was monitored until it had reached approximately 0.2, then nitric oxide saturated water was added, at the concentration stated, to one culture while the other was left as an unsupplemented control. Addition of the same concentration of NO was repeated at 30 minute intervals. The optical density at 650 nm was monitored for several hours following the initial NO pulse.

Growth with 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

Cells were grown as previously described (see above). 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) at a concentration of 1 mM or an equal volume of ethanol as a control was added to

one of each culture with nitrate or nitrite. The cell density was measured using a Corning colorimeter 252 and a 540 nm green filter. Colorimeter readings were taken every 60 minutes for 4 hours in those cultures without PTIO. Cultures with PTIO were spun down after 4 hours and resuspended in supplemented minimal medium. A reading was taken in the colorimeter. In an alternative experiment involving PTIO, nitrate or nitrite was added as usual then PTIO was added 2 hours later, 2 hours following this addition cells were collected by centrifugation and resuspended as before.

β-galactosidase assays

For β-galactosidase assays the method described by Jayaraman *et al.,* (1987) was used. Cells were grown in MS medium supplemented with 5 % LB, 0.4 % glycerol, 20 mM TMAO, 20 mM sodium fumarate and 35 μg ml^{-1} tetracycline. All cultures were started with 2 % (v/v) (unless otherwise stated) aerobic innocula grown in LB overnight with aeration at 37 °C. Anaerobic cultures were incubated statically at 37 °C either in 50 or 100 ml flasks filled with 50 or 100 ml of medium. Cultures were monitored until the optical density at 650 nm had reached approximately 0.2. A 1 ml sample of culture was lysed using 30 μl of 1 % (v/v) sodium deoxycholate and 30 μl toluene. The toluene was evaporated by aeration at 37 °C for 20 minutes. The lysate was either diluted 2-5 fold in 1xA or left undiluted. A 100 μl sample of lysate was added to 1.9 ml of Z buffer and then prewarmed at 37 °C. A volume of 0.5 ml of 13 mM ortho-nitrophenyl β-D-galactopyranoside (ONPG) was added, and the reaction was incubated at 37 °C until the colour was visibly yellow. The reaction was stopped after a known amount of time by adding 1 ml of 1 M sodium carbonate. The absorbance of the mixture at 420 nm was measured. The β-galactosidase

activity (nanomoles of ONPG hydrolysed min^{-1}.mg dry mass cells)$^{-1}$ was calculated using the formula below, where d is the dilution factor of the lysate, 2.5 is the coefficient used to convert absorbance at 650 nm into mg dry mass cells, 3.5 is the total reaction volume in ml, T is the incubation time of the reaction in min, 4.5 is the molar extinction coefficient for ortho-nitrophenol and v is the volume of lysate added in ml:

$$\text{Activity} = \frac{\text{Absorbance (420 nm)}}{\text{Absorbnce (650 nm)}} \text{ x } \frac{1000 \text{ x d x } 2.5 \text{ x } 3.5}{\text{T x } 4.5 \text{ x v}}$$

β-galactosidase assays in response to nitrate or nitrite

Purified transformants were grown anaerobically on minimal medium supplemented with 5 % LB, 0.4 % glycerol, 20 mM TMAO, 20 mM sodium fumarate and 35 µg ml^{-1} tetracycline. All cultures were started with 2 % (v/v) (unless otherwise stated) aerobic innocula grown in LB overnight with aeration at 37 °C. Anaerobic cultures were incubated statically at 37 °C either in 50 or 100 ml flasks filled with 50 or 100 ml of medium. Cultures were monitored until the optical density at 650 nm had reached approximately 0.2, then 5 mM nitrate or either 0.5 mM or 2.5 mM nitrite was added to one culture while the other was left as an unsupplemented control. The optical density at 650 nm was monitored for several hours following the nitrate or nitrite pulse. Samples of 1 ml were taken every 60 minutes, and were lysed and assayed for β-galactosidase activity.

β-galactosidase assays in response to nitric oxide

Purified transformants were grown anaerobically on minimal medium supplemented with 5 % (v/v) LB, 0.4 % (v/v) glycerol, 20 mM TMAO, 20 mM

sodium fumarate and 35 µg ml^{-1} tetracycline. All cultures were started with 2 % (unless otherwise stated) aerobic innocula grown in LB overnight with aeration at 37 °C. Anaerobic cultures were incubated statically at 37 °C either in 50 or 100 ml flasks filled with 50 or 100 ml of medium. Cultures were monitored until the optical density at 650 nm had reached approximately 0.2, then 1 or 5 µM NOSW was added to one culture while the other was left as an unsupplemented control. The same concentration of NO was added every 30 minutes. The optical density at 650nm was monitored for several hours following the initial NO pulse. Samples of 1 ml were taken every 60 minutes, and were lysed and assayed for β-galactosidase activity.

Growth of bacteria and release of enzyme for aconitase and fumarase assays

A single colony or the required strain was used to inoculate 1 ml of LB which was incubated at 37 °C for 5-8 hours with aeration. This 1 ml culture was used to inoculate 100 ml of minimal medium in a 100 ml flask, this was incubated statically at 37 °C overnight. This 100 ml culture was used to inoculate 1 L minimal medium supplemented with 5 mM nitrate. This 1 L culture was incubated statically at 37 °C for 4 hours. The cells were harvested by centrifugation at 6000 g for 10 min, resuspended in 15 ml of 50 mM phosphate buffer, and harvested again by centrifuging at 6000 g for 10 min. Cells were resuspended in 6 ml of phosphate buffer. The cells were disrupted by French pressing to release the enzymes. Following French pressing the cells were centrifuged at 40,000 g for 1 hour to remove the cell debris. The supernatant was kept and the pellet discarded.

Aconitase assay

The spectrophotometer used for the assay was the Helics Omega UV-VIS spectrophotometer from Thermo Scientific. The recording was set for an assay time of 5 minutes. An assay mixture containing 918 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 $NADP^+$ per 1 ml was added to a 1 ml cuvette. The assay mixture was incubated at 25 °C. The cuvette containing the assay mixture was used to zero the spectrophotometer. The spectrophotometer measured the absorbance of NADPH at 340 nm and the reaction was started by the addition of 50 µl of the supernatant containing the enzyme. The rate of increase in absorbance (A) over time is the rate of the reaction catalysed by aconitase. The units are $\Delta A\ min^{-1}$.

Fumarase assay

The spectrophotometer used for this assay was the same as for the aconitase assay. The recording was set for an assay time of 1 minute. Reagents were added to a 1 ml cuvette, 880 µl of 50 mM phosphate buffer (incubated at 25 °C), 5 µl of 40 mg ml^{-1} NAD^+ (incubated on ice), 50 µl of supernatant (incubated on ice), 50 µl of 0.1 M sodium glutamate (incubated on ice) and 5 µl of 0.3 mg ml^{-1} glutamate oxaloacetate aminotransferase (incubated on ice). This mixture was used to zero the spectrophotometer. The spectrophotometer measured the absorbance of NADH at 340 nm. The reaction was started by the addition of 10 µl of sodium fumarate. The rate of increase in absorbance is the rate of the reaction catalysed by fumarase. The units are $\Delta A\ min^{-1}$.

Folin assay to determine protein concentration

The supernatants used for both of the above assays were also assayed to determine the concentration of protein. The supernatants were diluted 1 in 10 with distilled water. BSA at 1 mg ml^{-1} was used as a standard. Either 50 or 100 µl of diluted supernatant or standard was added to a clean dry test tube and the volume made up to 600 µl with distilled water. To each sample in triplicate, 3 ml of Folin A solution was added, tubes were incubated at 37 °C for 10 minutes. Then 0.3 ml of phenol reagent was added to each tube, and the tubes were further incubated at 37 °C for 30 minutes. The absorbance at 750 nm was measured.

Growth and preparation of bacteria for use in the nitric oxide electrode

Bacteria were grown in MS supplemented with 5 % (v/v) LB, 0.4 % (v/v) glycerol, 20 mM TMAO, 20 mM sodium fumarate and when indicated 2.5 mM sodium nitrite or 5 or 20 mM sodium nitrate. Cultures were started with 10 % (v/v) inocula that had been grown at 37 ˚C with aeration for at least 4 hours. Anaerobic cultures were incubated statically overnight at 30 ˚C in flasks filled with 500 ml to 2000 ml of medium. The optical density at 650 nm was monitored until it had reached 0.6 to 0.8, and the bacteria were collected by centrifugation (8000 g, 2 min, 4 ºC). The bacteria were resuspended in 10 ml phosphate buffer and were homogenised. The washed bacteria were collected by centrifugation (3000 g, 3 min) and resuspended in phosphate buffer to give an optical density at 650 nm between 70 and 90. The bacteria were kept on ice until required.

NO electrode assays

An Oxytherm electrode control unit was used with an S1/MINI Clark type electrode disc and the Oxygraph Plus data acquisition software (Hansatech Instruments, Norfolk, UK). The electrolyte used was saturated KCl solution. To calibrate for the bottle of NOSW being used, 1788 µl degassed assay buffer, 32 µl of 1 M glucose, 20 µl of 0.4 U μl^{-1} glucose oxidase and 10 µl of 4 U μl^{-1}catalase were added to the electrode chamber using gas-tight Hamilton syringes. The trace was left to stabilise at zero. A volume of 150 µl of 2 mM NOSW was added and the stability of the trace was checked to ensure there was no rate of NO reduction when bacteria were absent. The amplitude of the reading given was recorded. The electrode chamber was washed once with ethanol, and then twice with distilled water. Then 32 µl of 1 M glucose, 20 µl of 0.4 U μl^{-1} glucose oxidase, 10 µl of 4 U μl^{-1} catalase and 100, 200 or 500 µl of bacterial suspension with the appropriate volume of assay buffer to give a final volume of 2 ml (after the addition of NO) was added to the electrode chamber. The trace was left to stabilise at zero, then NO reduction was started by the addition of 25, 50, 75, 100 or 150 µl of 2 mM NOSW. The rate of NO reduction (nanomoles of NO reduced min^{-1} .mg dry cell $mass^{-1}$) was calculated using the equation below:

$$Rate = \frac{slope\ x\ \left(\frac{N}{amplitude}\right)}{(V\ x\ 100\ x\ Absorbance\ (650\ nm)\ x\ 0.4)/2}$$

The slope is the initial rate of NO reduction as given by the ‘Rate Measurement’ function of the Oxytherm electrode control unit, N is the volume of NOSW being

used, ‘amplitude’ is the increase in arbitrary rate caused by N µl of NOSW in the electrode chamber alone, and the denominator represents the biomass of bacteria in the chamber: For the biomass value, V (ml) is the volume of cell suspension used, 100 x OD_{650} is required as the OD_{650} reading taken is a 1:100 dilution, 0.4 is the coefficient to convert absorbance at 650 nm into mg dry cell mass and 2 is the total volume in the electrode chamber in ml.

Increased sensitivity NO electrode assays

An alternative, more sensitive, electrode was used for some experiments to measure the rate of NO reduction. A modified Clark-type oxygen electrode system (Rank Brothers) harbouring an NO electrode (Precision Instruments ISO NOP sensor (2-mm diameter) was used with the NO sensor inserted through a tailor-made hole in the lid (Mills *et al*., 2001). The NO electrode was calibrated according to the manufacturer (Corker and Poole, 2003). Cells were prepared as previously described and the volumes of reagents added to the electrode chamber were the same as with the Oxytherm electrode except the cells are not added. The trace was left to stabilise at zero. Then Disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (commercial name PROLI NONOate) was added at a range of concentrations as a source of nitric oxide; the concentration of NO produced in the electrode is twice the concentration of PROLI NONOate added. Once the NO peak was seen to be stable, the bacterial cells were added to give a final volume of 2 ml as before and the rate of NO reduction was calculated in the same way.

CHAPTER 3 – RESULTS

Growth and NO generation - Introduction

The *E. coli* strain JCB5232 (*nirB, nrfA, norV*) and its derivatives (Table 3) were used for the following experiments. All are $NarG^+$. They differ in the mutations of the *hmp*, *hcp* and *ytfE* genes. These include JCB5210 (*hmp*), JCB5242 (*hcp*), JCB5250 (*hmp*, *hcp)*, JCB5260 (*hcp*, *hmp*, *ytfE)* and JCB5257 (*hmp*, *ytfE*).

Previous experiments by Claire Vine (2011) established that growth of strain JCB5250, deficient in all known or suspected NO reductases (*nirB*, *norVW*, *nrfA* and *hmp*) and *hcp*, was inhibited in the presence of nitrosative stress compared to an isogenic hcp^+ strain, JCB5210. This suggested that Hcp plays a role in the nitrosative stress response. However at the onset of this work the exact role of Hcp was unclear. Curiously, a further mutation in *ytfE* (strain JCB5260), partially restored the attenuated growth observed for the *hcp* mutant (strain JCB5250). It is thought that this is due to YtfE producing a toxic product, possibly during the repair of iron-sulphur clusters damaged by nitrosative stress. It was thought that Hcp might remove this toxic product.

The effect of nitrite on growth of strains susceptible to nitrosative stress

To confirm the effect of nitrite on strains proposed to be sensitive to nitrosative stress the parent strain for these experiments, JCB5232, was grown anaerobically in the presence and absence of 0.5 mM nitrite. JCB5232 grew well under both conditions (data not shown). There was no inhibition of growth in the presence of nitrite even though the strain is deficient in many known NO reductases (Table 2.1).

The effect of an *hmp* mutation on sensitivity to nitrosative stress

To investigate the effect of an *hmp* mutation on sensitivity to nitrite, growth of the *hmp* deletion strain (JCB5210) in the presence and absence of 0.5 mM nitrite was examined. The JCB 5210 strain grew well under both conditions and the presence of nitrite had no significant impact on cell growth despite this strain being deficient in all known NO reductase systems (Figure 3.1).

The effect of an *hcp* mutation on sensitivity to nitrosative stress

To investigate the effect of an *hcp* mutation on sensitivity to nitrite, strain JCB5250 (*hmp*, *hcp*) was grown in the presence and absence of 0.5 mM nitrite. JCB5250 grew well in the absence of nitrite, but in the presence of nitrite growth was inhibited. An *hcp* mutation resulted in inhibited growth in the presence of nitrite (Figure 3.2a).

To investigate whether Hmp could protect against nitrosative stress in an hcp^- background, strain JCB5142 (hmp^+, *hcp*) was grown under similar conditions. JCB5242 also grew well in the absence of nitrite, but growth was still inhibited to the same extent by nitrite. The presence of a functional *hmp* gene did not restore resistance to nitrosative stress (Figure 3.2b).

Evidence that the toxic product generated by YtfE is not Fe^{2+}

Previous work by the Saraiva group suggested that the toxic product produced by YtfE might be Fe^{2+}. The YtfE protein has been shown to be critical for the repair of iron-sulphur clusters damaged by nitrosative stress. It has also been

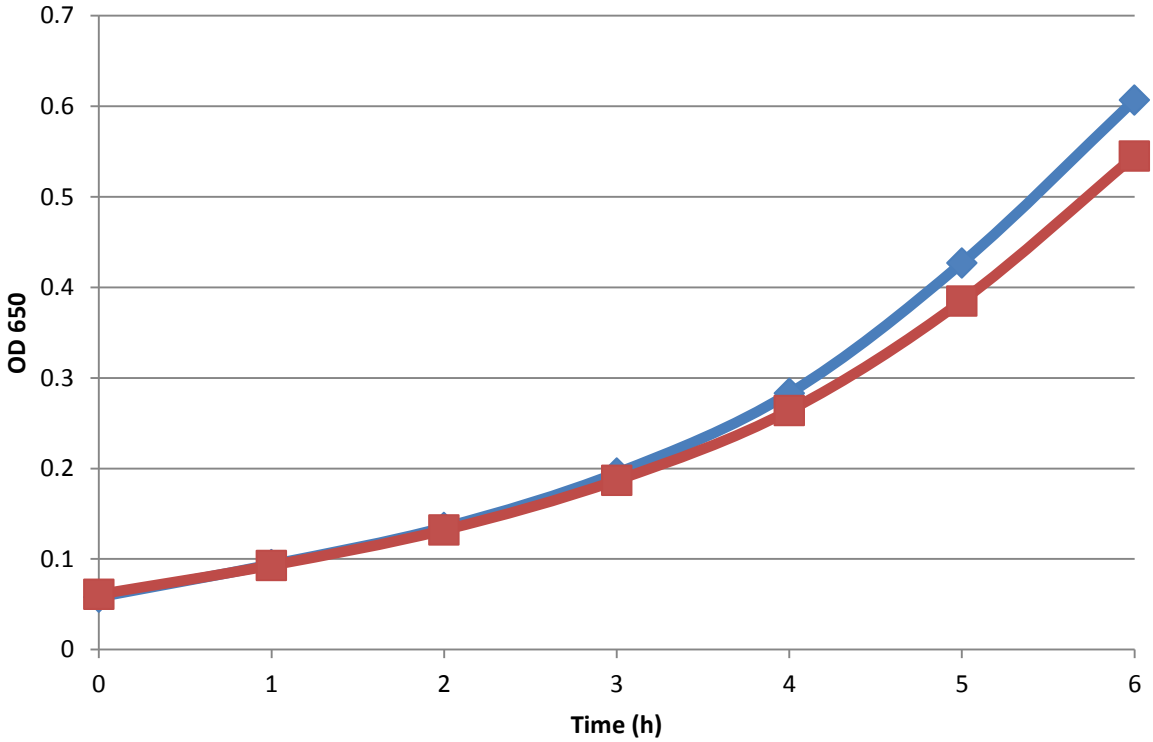


Figure 3.1. Effect of nitrite on growth of the *hmp* mutant.

Strain JCB5210 (*hmp*$^-$) was grown anaerobically in the absence of nitrite (blue line) and with 0.5 mM nitrite (red line). The x-axis shows the time in hours following the addition of nitrite.

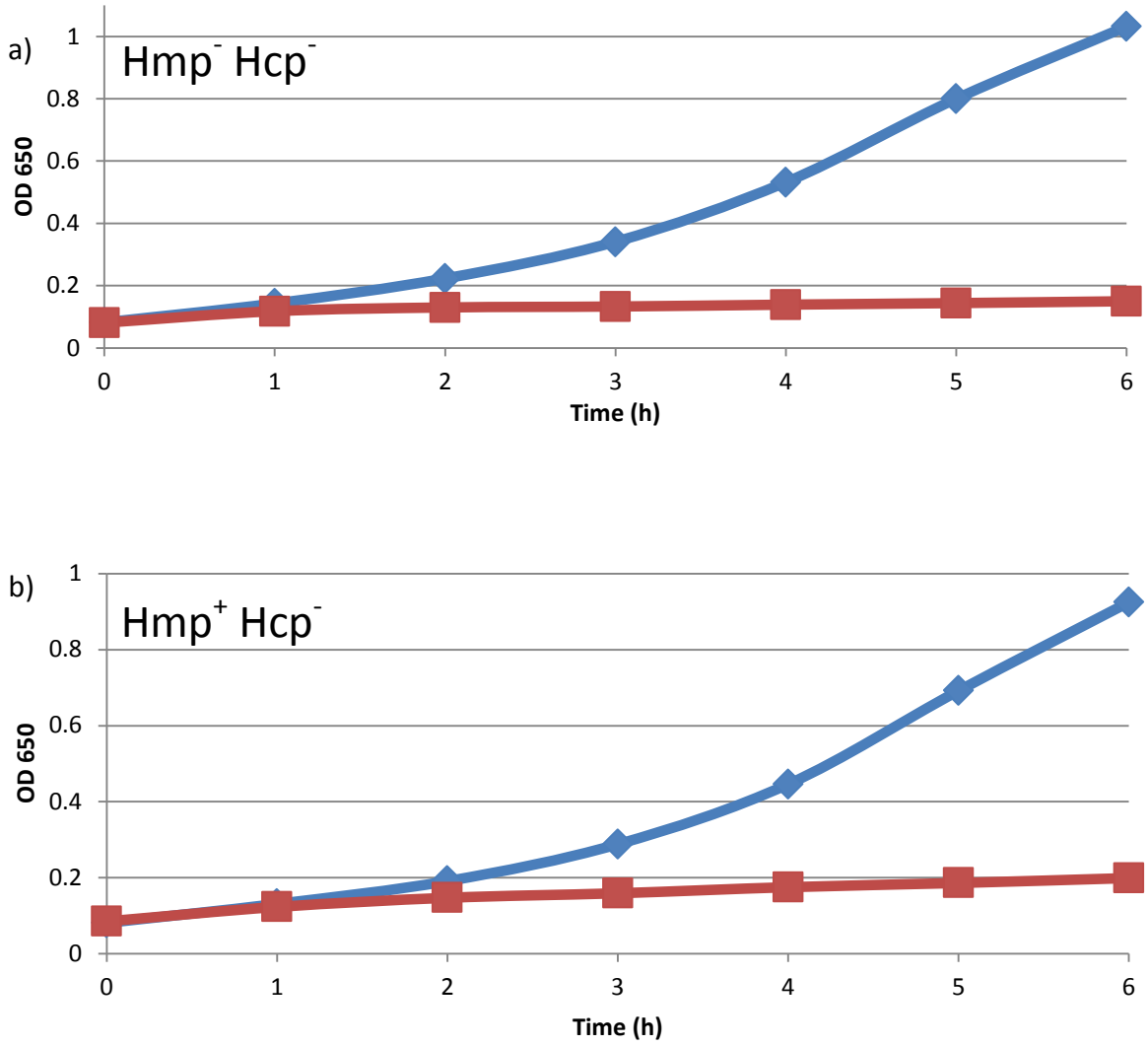


Figure 3.2. Effect of nitrite on growth of the *hcp* mutant.

Strains JCB5242 (*hcp*$^-$) and JCB5250 were grown anaerobically in the absence of nitrite (blue line) and with 0.5 mM nitrite (red line). Growth curves for the hmp, hcp, and hcp deletion strains are shown in panels a) and b) respectively. The x-axis shows the time in hours following the addition of nitrite.

suggested that YtfE is involved in the recruitment and integration of ferrous ion (Fe^{2+}) into iron- sulphur centres. If Fe^{2+} is responsible for the inhibited growth seen in the presence of nitrite then 2,2-2,2-dipyridyl, which chelates Fe^{2+} in a biologically inert complex, should relieve this inhibited growth. The effects of 2,2-dipyridyl on isogenic pairs of *ytfE* and *ytfE*$^{+}$ strains during growth in the presence of nitrite were therefore compared.

The effect of 100 µM 2,2-dipyridyl on growth of the *ytfE*$^{+}$ strains, JCB5210 and JCB5250, were compared (Figure 3.3 a and b). Growth of both strains in the presence of 2,2-dipyridyl was strongly inhibited. Furthermore, there was no evidence that 2,2-dipyridyl relieved growth inhibition by nitrite of the *hcp* mutant.

The effect of 2,2-dipyridyl on growth of strains deficient in YtfE were also investigated (Figure 3.3c and 3.3d). As with the *ytfE*$^{+}$ strains, growth was strongly inhibited in the presence of 2,2-dipyridyl irrespective of whether a functional *hcp* gene is present or absent. Note that the YtfE^{-} strains were slightly less sensitive to nitrosative stress and 2,2-dipyridyl than the isogenic YtfE^{+} strains.

In order to investigate whether the same effects were seen with nitrate as the indirect source of nitrosative stress, the same strains as above were grown in the absence of nitrosative stress, in the presence of 5 mM nitrate and in the presence of 5 mM nitrate and 100 µM 2,2-2,2-dipyridyl. The *hcp*$^{+}$ *ytfE*$^{+}$ strain, JCB5210 (*hmp*), grew well in the absence of nitrate. Growth was stimulated in the presence of nitrate, but in the presence of nitrate and 2,2-dipyridyl, growth was partially inhibited (Figure 3.4 a). As seen previously in the presence of

nitrite, growth of this control strain was inhibited by the presence of an iron chelator. Strain JCB5250 (*hmp, hcp*) also grew well in the absence of nitrosative stress.

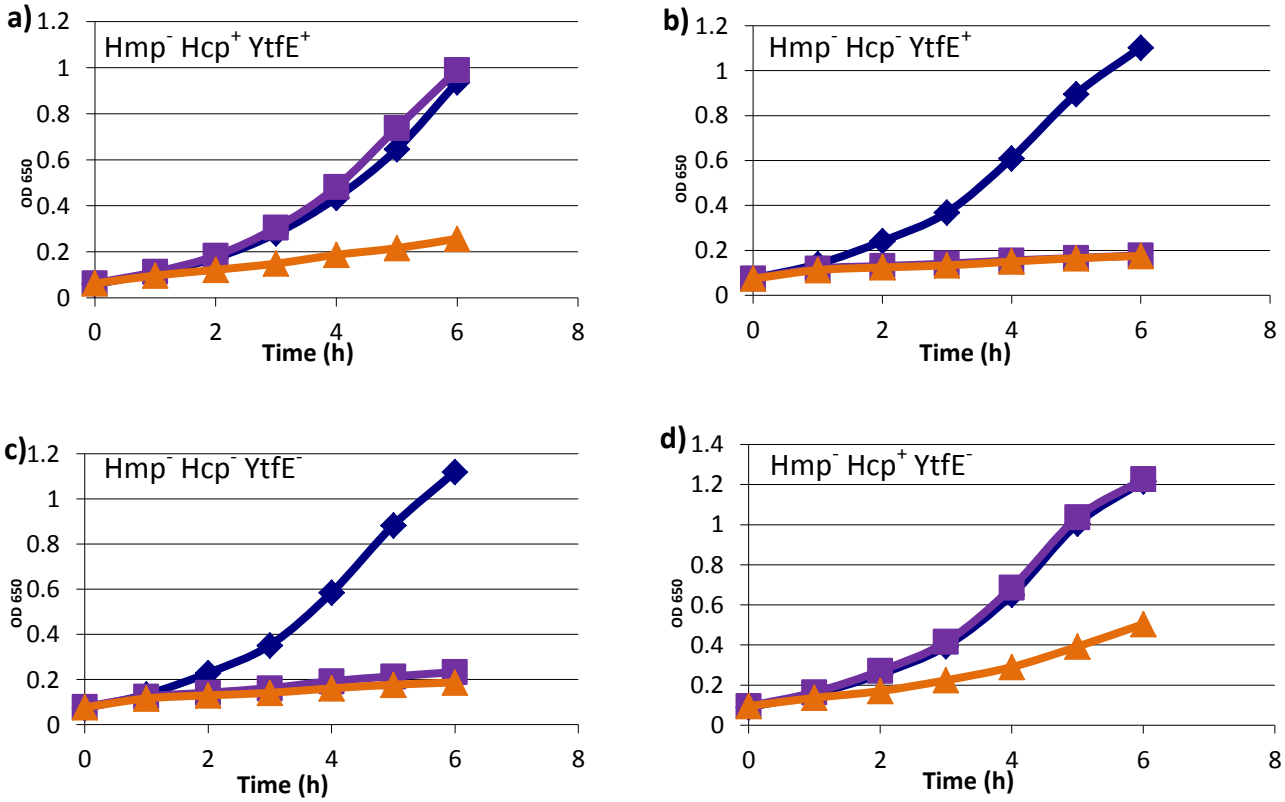


Figure 3.3 Effect of nitrite 2,2-dipyridyl on growth of strains sensitive to nitrosative stress.

Strains sensitive to nitrosative stress, with JCB5210 as a control, were grown in the absence of nitrite and the iron-chelator 2,2-2,2-dipyridyl (blue line.) With 0.5 mM nitrite (purple line) and with both 0.5 mM nitrite and 100 µM 2,2-2,2-dipyridyl (orange line). a) JCB5210 (*hmp*$^-$), b) JCB5250 (*hmp*$^-$ *hcp*$^-$), c) JCB5260 (*hmp*$^-$ *hcp*$^-$ *ytfE*$^-$) d) JCB5257 (*hmp*$^-$ *ytfE*$^-$). The x-axis shows the time in hours following the addition of nitrite and 2,2-2,2-dipyridyl.

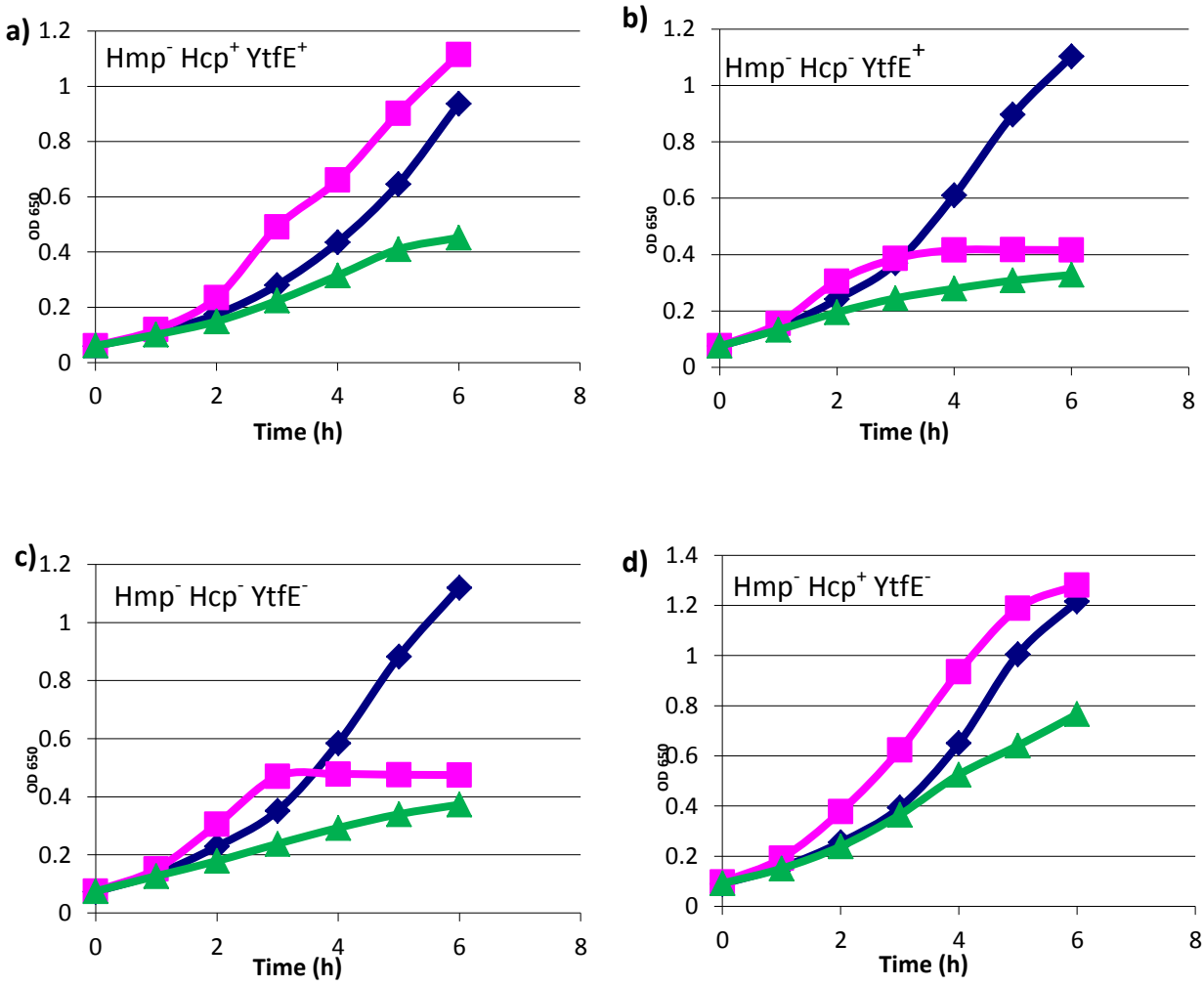


Figure 3.4 Effect of nitrate and 2,2-2,2-dipyridyl on growth of stains sensitive to nitrosative stress.

Strains sensitive to nitrosative stress, with JCB5210 as a control, were grown in the absence of nitrate and 2,2-dipyridyl (blue line) with 5 mM nitrate (pink line) and with both 5 mM nitrate and 100 μM 2,2-2,2-dipyridyl (green line). a) JCB5210 (*hm-*), b) JCB5250 (*hmp- hcp-*), c) JCB5260 (*hmp- hcp- ytfE-*) d) JCB5257 (*hmp- ytf-*). The x-axis shows the time in hours following the addition of nitrite and 2,2-2,2-dipyridyl.

Initially this strain also grew well in the presence of nitrate but after 2 to 3 hours following the addition of nitrate growth was inhibited. This was most likely due to the reduction of nitrate to nitrite, which was then reduced further to nitric oxide. Growth of this strain was further inhibited in the presence of nitrate and 2,2-dipyridyl without the initial phase of growth (Figure 3.4b).

The corresponding YtfE$^-$ strain JCB5260 (*hmp, hcp, ytfE*) again grew well in the absence of nitrate and 2,2-dipyridyl. Again the strain initially grew well in the presence of nitrate, but after 2 to 3 hours, growth was inhibited. Growth was further inhibited in the presence of nitrate and 2,2-dipyridyl, again without the initial growth (Figure 3.4c). JCB5257 (*hmp, ytfE*) grew well in the absence of nitrate. Growth was stimulated in the presence of nitrate. In the presence of nitrate and 2,2-dipyridyl growth was only partially inhibited (Figure 3.4d). In conclusion, no evidence was obtained from these experiments that 2,2-dipyridyl reversed growth inhibition due to nitrosative stress.

The effect of Fe^{2+} on growth of strains sensitive to nitrosative stress

As 2,2-dipyridyl, a Fe^{2+} chelator, did not relieve inhibition of growth due to the presence of nitrosative stress, and appeared to further inhibit growth, Fe^{2+} itself was added. As the chelation of iron appeared to be detrimental to the growth of these strains, the addition of Fe^{2+}should either have no effect on growth or stimulate growth. To investigate if Fe^{2+}stimulates growth of strains inhibited by nitrosative stress, these strains were grown under various conditions. Strain JCB5210, as an *hcp*$^+$ control, and strains sensitive to nitrosative stress were grown in the absence of nitrite and ferrous sulphate, in the presence of 0.5 mM

nitrite and in the presence of 0.5 mM nitrite and 100 μM ferrous sulphate. The *hcp*$^{+}$ *ytfE*$^{+}$ strain, JCB5210 (*hmp*), grew well in the absence of nitrosative stress and in the presence of nitrite (Figure 3.5a). The presence of nitrite and ferrous sulphate had no effect on growth of this control strain, growth was neither inhibited nor stimulated. The Hmp^{+} Hcp^{-} strain, JCB5242, also grew well in the absence of nitrite, but growth was inhibited in the presence of nitrite. In the presence of nitrite and ferrous sulphate growth was partially restored (Figure 3.5 b). Theinhibited growth seen in the presence of nitrosative stress can be partially relieved by the addition of ferrous sulphate. The Hmp^{-} Hcp^{-} strain, JCB5250, also grew well in the absence of nitrosative stress, but again growth was inhibited in the presence of nitrite. In the presence of nitrite and ferrous sulphate growth was slightly better but not as clearly restored as in the *hmp*$^{+}$ strain (Figure 3.5c). The corresponding YtfE^{-} strain, JCB5260 (*hmp, hcp, ytfE*), also grew well in the absence of nitrite, but again growth was inhibited in the presence of nitrite. In the presence of nitrite and ferrous sulphate growth was again partially restored (Figure 3.5d). The inhibited growth seen in strains deficient in a functional *hcp* gene can be partially restored by the addition of ferrous sulphate.

To investigate if higher concentrations of ferrous sulphate would result in greater relief of growth inhibition, strains previously shown to be recovered by ferrous sulphate were grown in the absence of nitrite, in the presence of 0.5 mM nitrite and in the presence of 0.5 mM nitrite and various concentrations of ferrous sulphate (100 μM, 200 μM or 500 μM). The *hcp*$^{+}$ control strain, JCB5257 (*hmp, ytfE*), grew well in the absence of nitrite and growth was not inhibited in the presence of nitrite. The addition of 100, 200 or 500 μM ferrous sulphate did

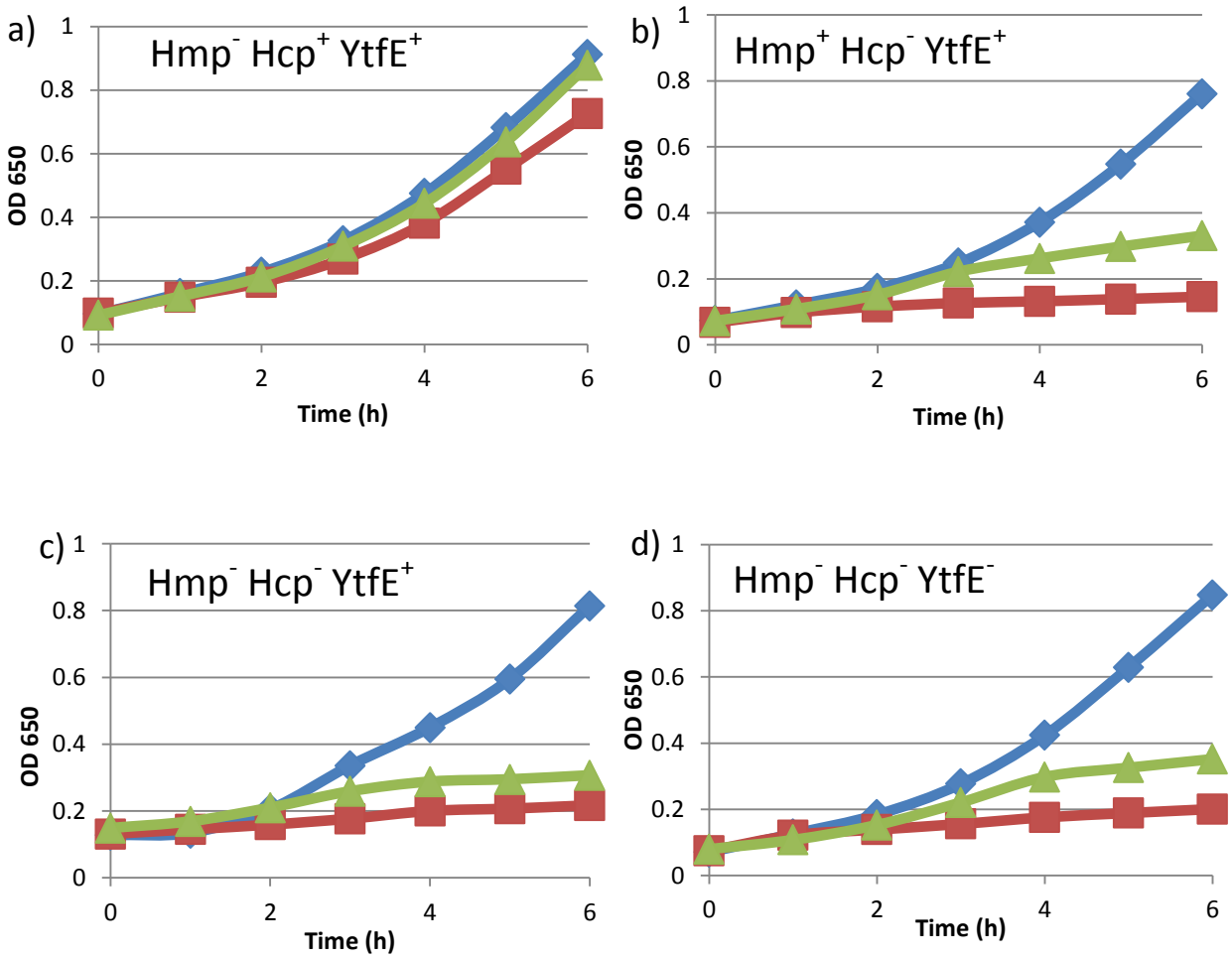


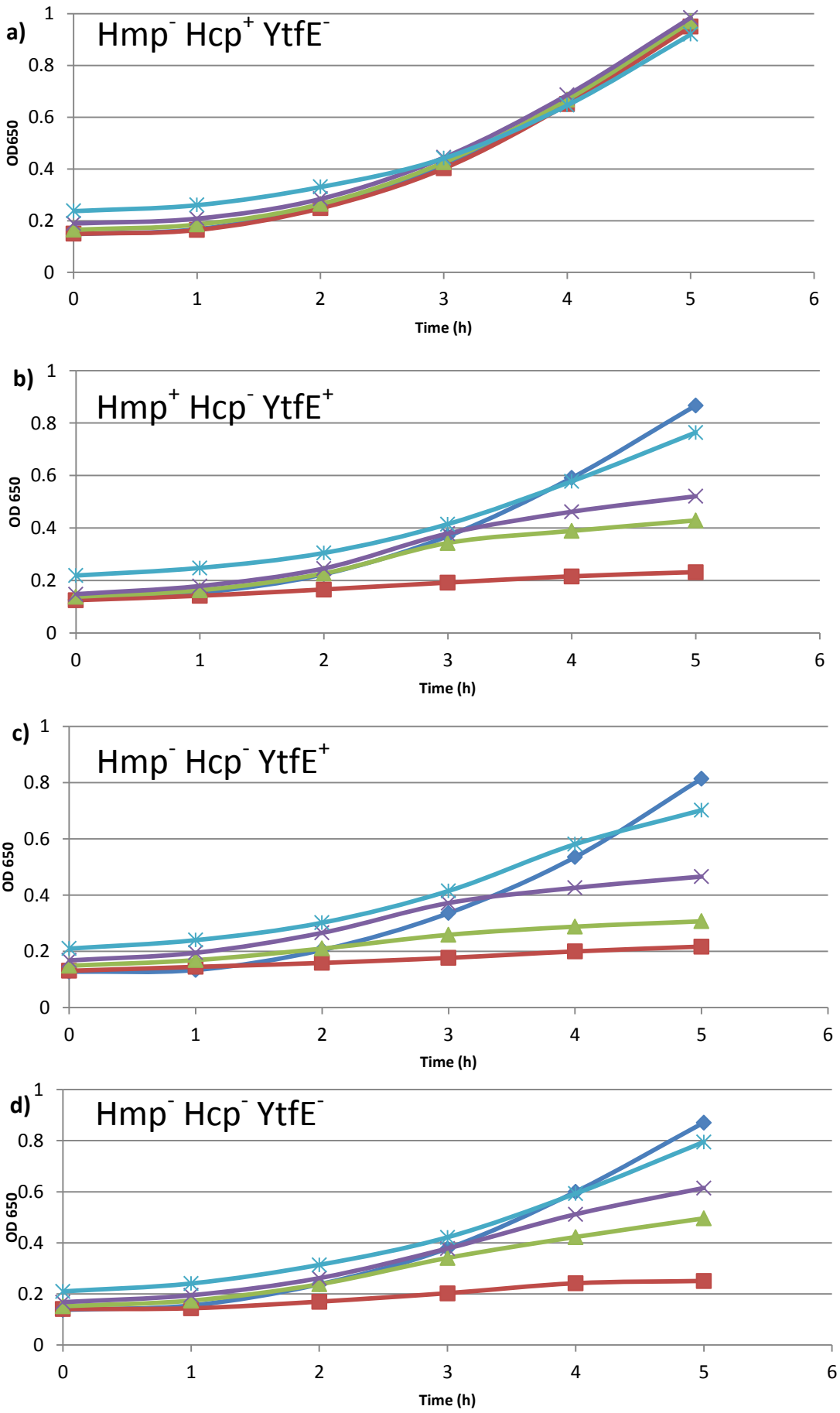
Figure 3.5 Effect of nitrite and ferrous sulphate on growth of strains sensitive to nitrosative stress.

Strains sensitive to nitrosative stress, with JCB5210 as a control, were grown in the absence of nitrite and ferrous sulphate (blue line), with 0.5 mM nitrite (red line) and with both 0.5 mM nitrite and 100 μM ferrous sulphate (green line). a) JCB5210 (*hmp*$^-$), b) JCB5242 (*hcp*$^-$) c) JCB5250 (*hmp*$^-$ *hcp*$^-$), d) JCB5260 (*hmp*$^-$ *hcp*$^-$ *ytfE*$^-$). The x-axis shows the time in hours following the addition of nitrite and ferrous sulphate.

not affect growth of this control strain (Figure 3.6a). An *hcp* mutant, JCB5242 (*hmp*$^{+}$), grew well in the absence of nitrosative stress, but growth was inhibited in the presence of nitrite. In the presence of nitrite and ferrous sulphate growth was restored in a dose-dependent manner, with 100 μM ferrous sulphate only partially restoring growth and with 500 μM ferrous sulphate almost completely relieving growth inhibition (Figure 3.6b). Another *hcp* mutant, JCB5250 (*hmp, hcp*), also grew well in the absence of nitrosative stress, but again growth was inhibited in the presence of nitrite. In the presence of nitrite and ferrous sulphate growth was again partially restored (Figure 3.6c). For this strain, addition of 100 μM ferrous sulphate was insufficient to fully restore growth when compared to an isogenic *hmp*$^{+}$ strain. However, with 500 μM ferrous sulphate present, the previous growth defect was almost completely relieved. This suggests Hmp may partially protect against nitrosative stress but only slightly. This is probably by removing small amounts of nitric oxide. The corresponding YtfE^{-} strain, JCB5260 (*hmp, hcp, ytfE*), also grew well in the absence of nitrite, but again growth was inhibited in the presence of nitrite. In the presence of nitrite and ferrous sulphate inhibition of growth was again relieved in a concentration-dependent manner (Figure 3.6d). As seen with the other two strains sensitive to nitrosative stress, ferrous sulphate at a concentration of 100 μM partially restores growth and at a concentration of 500 μM inhibition of growth by nitrite is almost totally relieved. Growth was restored as much with 100 μM ferrous sulphate in this strain as with the Hmp^{+} Hcp^{-} strain, JCB5242, suggesting that a *ytfE* mutation also partially protects against nitrosative stress. This observation is consistent with YtfE^{-} cells being unable to produce a cytotoxic species that may results from the action of the YtfE protein *in vivo*.

Figure 3.6 Effect of nitrite and ferrous sulphate on growth of strains sensitive to nitrosative stress.

Strains sensitive to nitrosative stress, with JCB5257 as a control, were grown anaerobically in the absence of nitrite and ferrous sulphate (blue line), with 0.5 mM nitrite (red line) and with 0.5 mM nitrite and 100 μM ferrous sulphate (green line), 200 μM ferrous sulphate (purple line) and 500 μM ferrous sulphate (light blue line). a) JCB5257 (*hmp*$^-$ *ytfE*$^-$) b) JCB5242 (*hcp*$^-$) c) JCB5250 (*hmp*$^-$ *hcp*$^-$), d) JCB5260 (*hmp*$^-$ *hcp*$^-$ *ytfE*$^-$). The x-axis shows the time in hours following the addition of nitrite and ferrous sulphate.

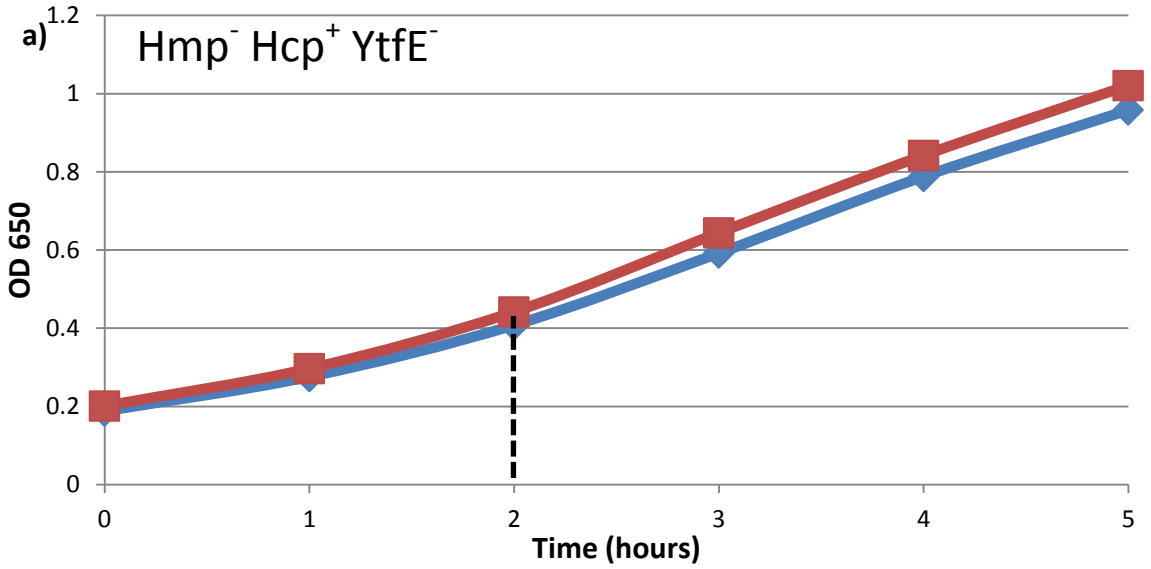

a)
Hmp- Hcp+ YtfE-
OD650
Time (h)
b)
Hmp+ Hcp- YtfE+
OD 650
Time (h)
c)
Hmp- Hcp- YtfE+
OD 650
Time (h)
d)
Hmp- Hcp- YtfE-
OD 650
Time (h)

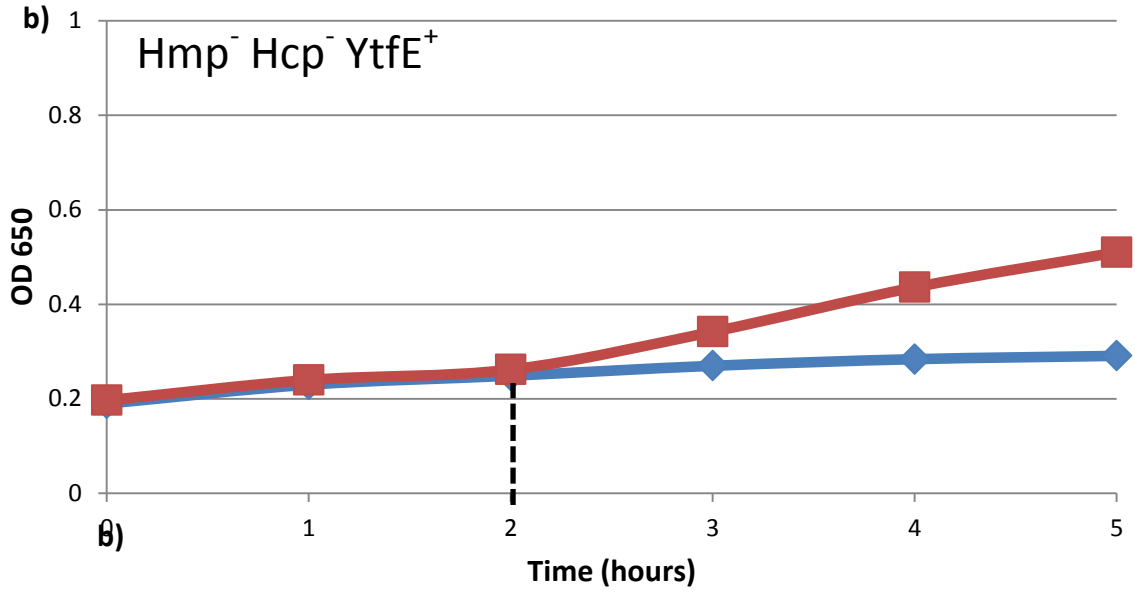
The previous experiments have shown that ferrous sulphate can recover the inhibited growth seen in an *hcp* mutant in the presence of nitrosative stress. To investigate whether the ferrous sulphate needs to be available at the time of initial nitrosative stress exposure or whether the addition of ferrous sulphate several hours following onset of nitrosative stress can produce the same effect the following experiment was designed. The Hcp^+ control strain, JCB5257 (*hmp, ytfE*), strain JCB5250 (*hmp, hcp*) and strain JCB5260 (*hmp, hcp, ytfE*), were grown in the presence of 0.5 mM nitrite, and in the presence of 0.5 mM nitrite with the addition of 200 µM ferrous sulphate 2 hours following addition of nitrite. Strain JCB5257 (*hmp, ytfE*) grew well in the presence of nitrite. There was no effect on growth by the addition of ferrous sulphate 2 hours following the addition of nitrite to this control strain (Figure 3.7a). Growth of strain JCB5250 (*hmp, hcp*) was inhibited in the presence of nitrite, but upon addition of ferrous sulphate 2 hours following the addition of nitrite, growth inhibition was relieved (Figure 3.7b). Therefore, ferrous sulphate was capable of relieving growth inhibition due to the presence of nitrite even after nitrosative stress has occurred and damaged internal proteins, 2 hours following addition of nitrite. The corresponding $YtfE^-$ strain, JCB5260 (*hmp, hcp, ytfE*) also showed inhibited growth in the presence of nitrite, but this inhibition was again relieved upon addition of ferrous sulphate 2 hours following the addition of nitrite (Figure 3.7 c). Ferrous sulphate is again capable of relieving growth inhibition. This suggests that ferrous ions are required for repair of damage and are not simply mopping up nitric oxide produced from reduction of nitrite.

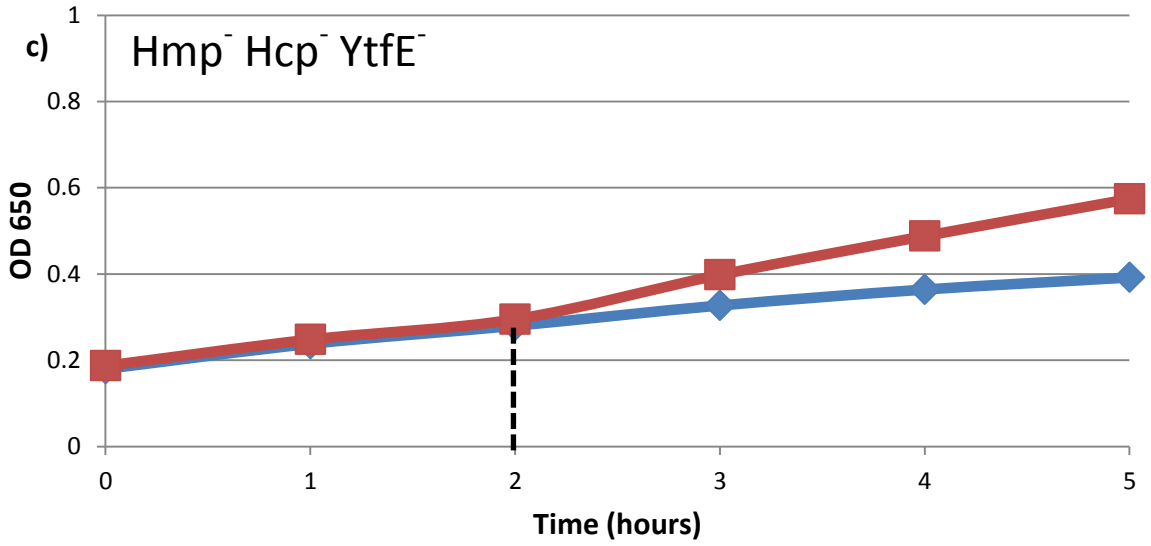
The addition of ferrous sulphate partially relieved the growth inhibition seen in *hcp* mutants due to the presence of nitrite. The effect of ferrous sulphate on the

Figure 3.7 Effect of nitrite and ferrous sulphate 2 hours following addition of nitrite on strains sensitive to nitrosative stress.

Strains sensitive to nitrosative stress, with JCB5257 as a control, were grown anaerobically in the presence of 0.5 mM nitrite (blue line) and with 0.5 mM nitrite and 200 μM ferrous sulphate (red line). a) JCB5257 (*hmp*$^-$ *ytE*$^-$) b) JCB5250 (*hmp*$^-$ *hcp*$^-$) c) JCB5260 (*hmp*$^-$ *hcp*$^-$ *ytfE*$^-$). The x-axis shows the time in hours following addition of nitrite. The dashed lines indicate the addition of ferrous sulphate for the red line.


a)
Hmp- Hcp+ YtfE-
OD 650
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Time (hours)


b)
Hmp- Hcp- YtfE+
OD 650
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Time (hours)


c)
Hmp- Hcp- YtfE-
OD 650
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Time (hours)

growth of an *hcp* mutant in the absence of nitrosative stress was then investigated. The *hcp* mutant strain, JCB5250 (*hmp, hcp*), and the *hcp*$^{+}$ control strain, JCB5210 (*hmp*), were grown in the absence of nitrosative stress and without ferrous sulphate, without nitrosative stress in the presence of 500 µM ferrous sulphate from the start of the experiment and without nitrosative stress with the addition of 500 µM ferrous sulphate 2 hours following the start of the experiment. The *hcp*$^{+}$ strain, JCB5210 (*hmp*), grew well in the absence of nitrosative stress and no effect on growth was seen by the addition of ferrous sulphate either at the start of the experiment or 2 hours later (Figure 3.8a). Growth of the *hcp* mutant strain, JCB5250 (*hmp, hcp*), was inhibited in the absence of nitrosative stress, but when ferrous sulphate was present from the start of the experiment growth inhibition was completely relieved. When ferrous sulphate was added 2 hours later growth inhibition was partially relieved (Figure 3.8b). Again this suggests that addition of ferrous sulphate can relieve the growth inhibition of an *hcp* mutant at the onset of stress or later, with the stress in this case being anaerobic growth, not nitrosative stress. This also supports the idea that ferrous ions are not simply mopping up nitric oxide to restore growth, as in these conditions NO is absent.

Evidence that the toxic product generated by YtfE is nitric oxide

Another possibility for the toxic product produced by YtfE is NO itself. PTIO is a stable radical scavenger of NO. It reacts with NO to form the corresponding imino nitroxides (PTI's) and nitrite. If NO is the toxic product, addition of PTIO should cause relief of the inhibited growth seen in an *hcp* mutant. To determine if NO is the toxic product generated by YtfE the isogenic set of strains were grown in the presence of 5 mM nitrate, in the presence of 5 mM nitrate and 1

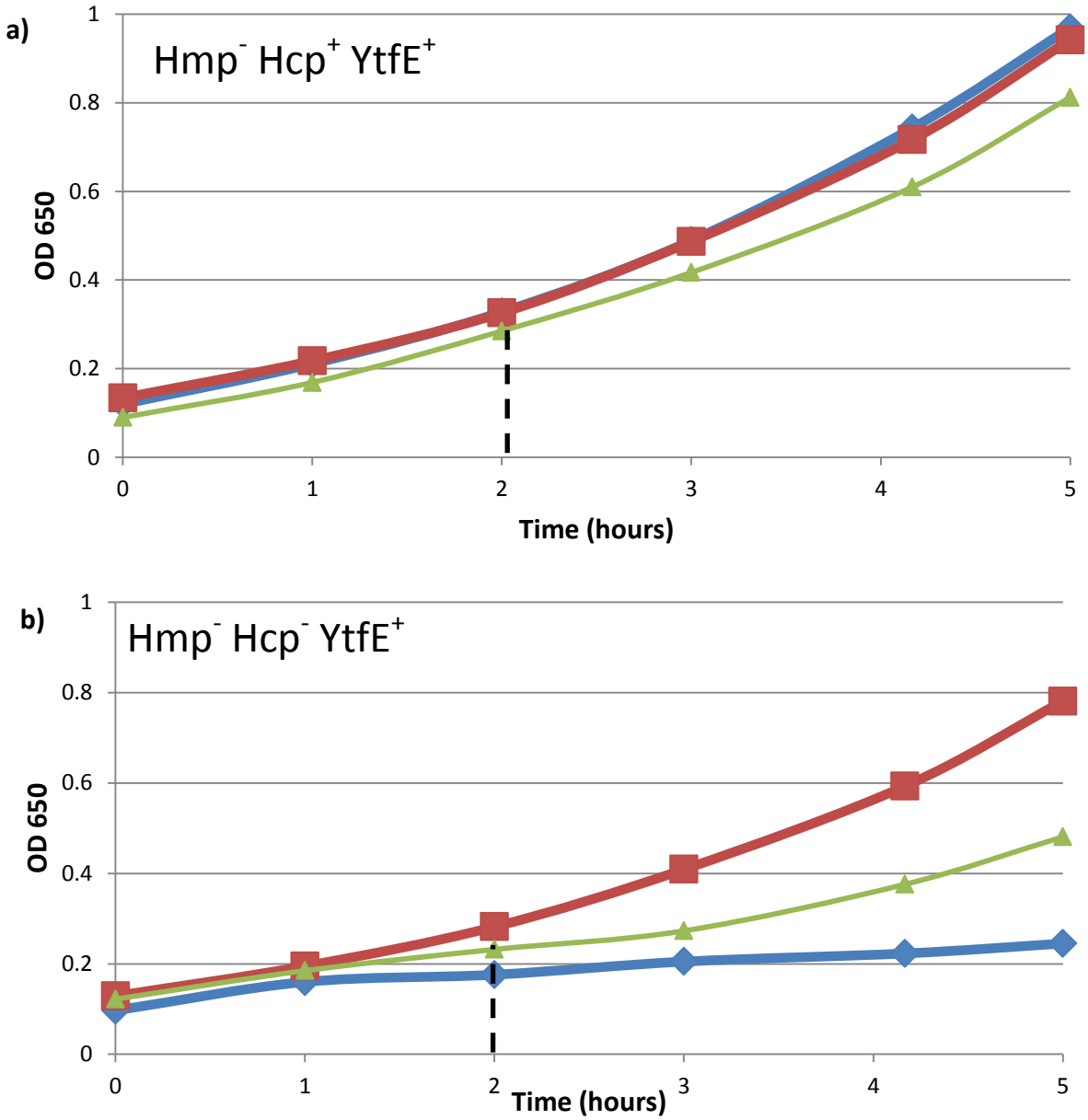


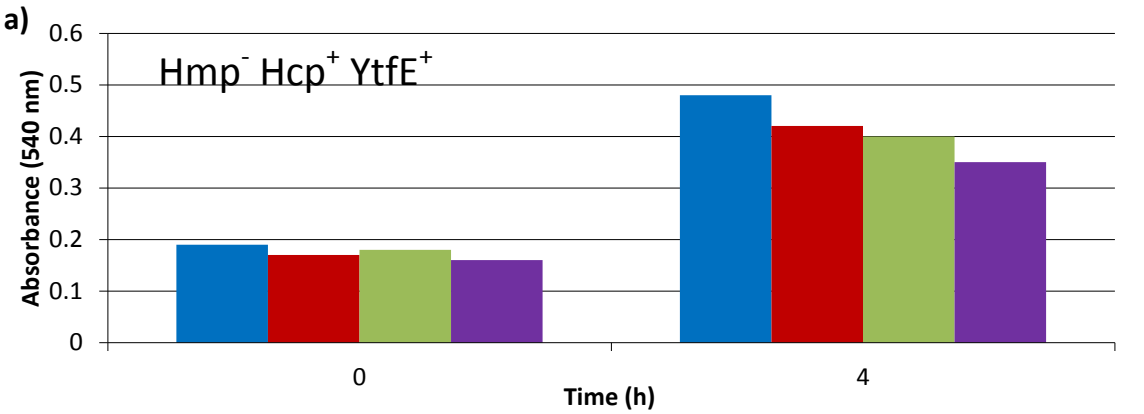
Figure 3.8 Effect of ferrous sulphate on an *hcp* mutant in the absence of nitrosative stress.

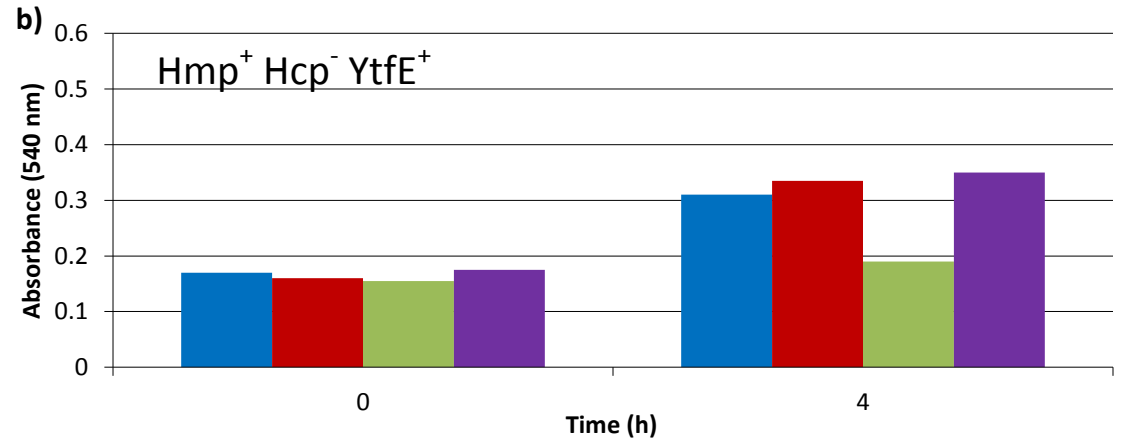
JCB5250 (*hmp*$^-$ *hcp*$^-$) (b) with JCB5210 as a control (a) were grown in the absence of nitrosative stress (Blue line) in the presence of 500 µM ferrous sulphate (Red line) and with the addition of 500 µM ferrous sulphate after 2 hours (Green line). The x-axis shows the time in hours from the start of the experiment. The dashed lines indicate the time of addition of ferrous sulphate for the green line.

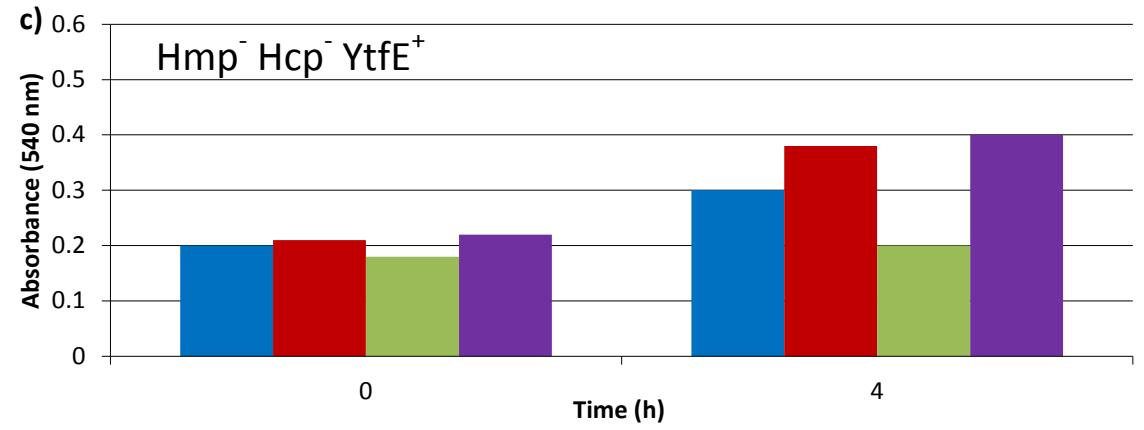
mM PTIO, in the presence of 0.5 mM nitrite and in the presence of 0.5 mM nitrite and 1 mM PTIO. The *hcp*$^{+}$ control strain, JCB5210 (*hmp*), grew well in the presence of nitrate and nitrite but showed slightly inhibited growth in the presence of either form of nitrosative stress and PTIO (Figure 3.9a). Another control *hcp*$^{+}$ strain, JCB5257 (*hmp*, *ytfE*), also grew well in the presence of nitrate and nitrite and were again slightly inhibited in the presence of either source of nitrosative stress and PTIO. This strain acts as an alternative control to the corresponding YtfE^{+} strain, JCB5210 (data not shown). Growth of the *hcp* mutant strain, JCB5242 (*hmp*$^{+}$), was inhibited in the presence of nitrate and was further inhibited in the presence of nitrite. In the presence of nitrate and PTIO, growth inhibition was slightly relieved compared to in the presence of nitrate alone. In the presence of nitrite and PTIO, growth inhibition was completely relieved compared to in the presence of nitrite alone (Figure 3.9b). PTIO, an NO scavenger, relieves the growth inhibition seen in the presence of nitrosative stress in an *hcp* mutant. This suggests that NO is responsible for the inhibited growth observed and therefore implies that the cytotoxic species produced by the action of YtfE *in vivo* is the NO free-radical. Growth of another *hcp* mutant strain, JCB5250 (*hmp*, *hcp*), was also inhibited in the presence of nitrate and was again further inhibited in the presence of nitrite. In the presence of nitrate and PTIO growth inhibition was more clearly relieved than in the corresponding Hmp^{+} strain. In the presence of nitrite and PTIO growth inhibition was again completely relieved (Figure 3.9c). This again suggests that NO inhibits growth in these strains. The corresponding YtfE^{-} strain, JCB5260 (*hmp*, *hcp*, *ytfE*), was also inhibited in the presence of nitrate and as for the previous two strains further inhibited by nitrite. In the presence of either source of nitrosative stress

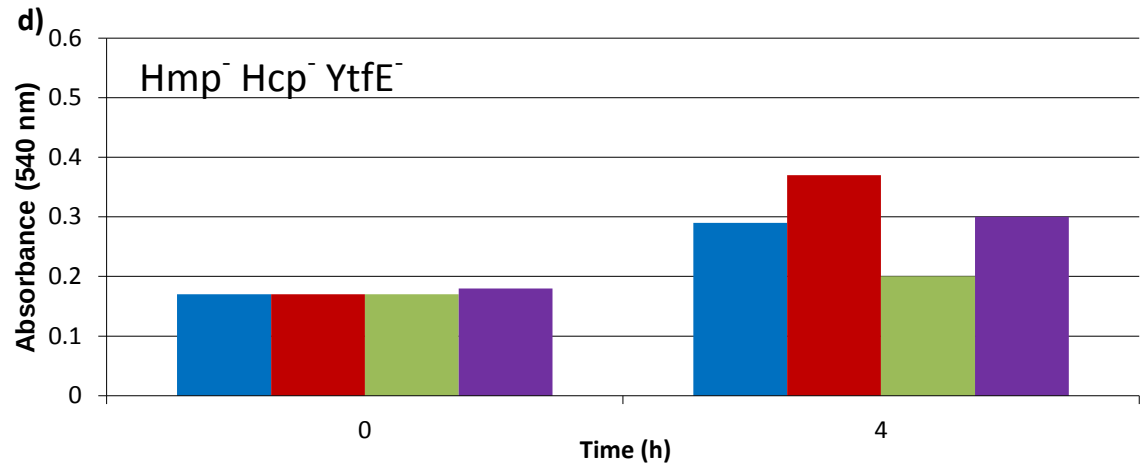
Figure 3.9 Effect of PTIO on growth inhibition in strains sensitive to nitrosative stress.

Strains sensitive to nitrosative stress, with hcp^+ strains, JCB5210 (*hmp*) and JCB5257 (*hmp*, *ytfE*) as controls, were grown anaerobically in the presence of 5 mM nitrate (blue bars), in the presence of 5 mM nitrate and 1 mM PTIO (Red bars), in the presence of 0.5 mM nitrite (Green bars) and in the presence of 0.5 mM nitrite and 1 mM PTIO (Purple bars). a) JCB5210 (*hmp*) b) JCB5242 (*hcp*), c) JCB5250 (*hmp*, *hcp*), d) JCB5260 (*hmp*, *hcp*, *ytfE*). This is representative data so no error bars are present.


a)
Hmp- Hcp+ YtfE+
Absorbance (540 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
Time (h)


b)
Hmp+ Hcp- YtfE+
Absorbance (540 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
Time (h)


c)
Hmp- Hcp- YtfE+
Absorbance (540 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
Time (h)


d)
Hmp- Hcp- YtfE-
Absorbance (540 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
Time (h)

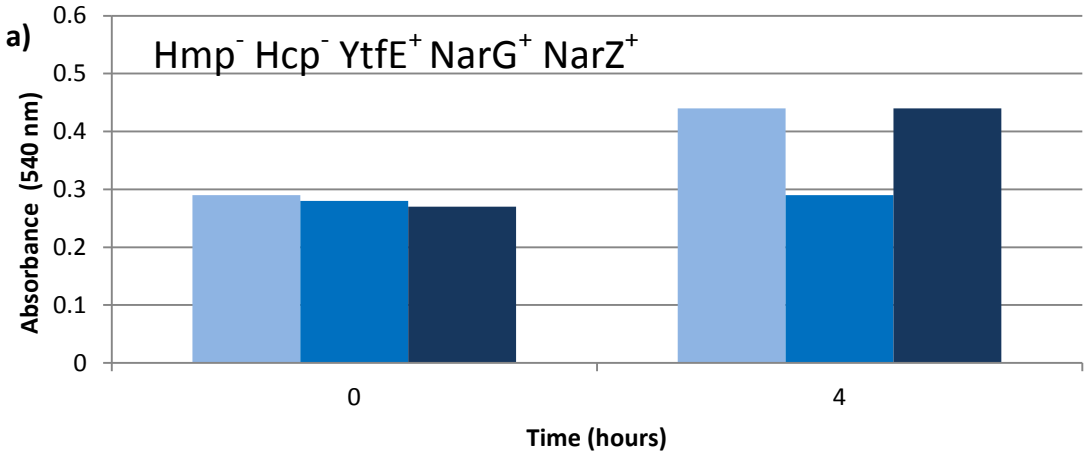
and PTIO growth inhibition was partially relieved compared to the source of stress alone (Figure 3.9d). This again suggests that NO is responsible for the poor growth in an *hcp* mutant.

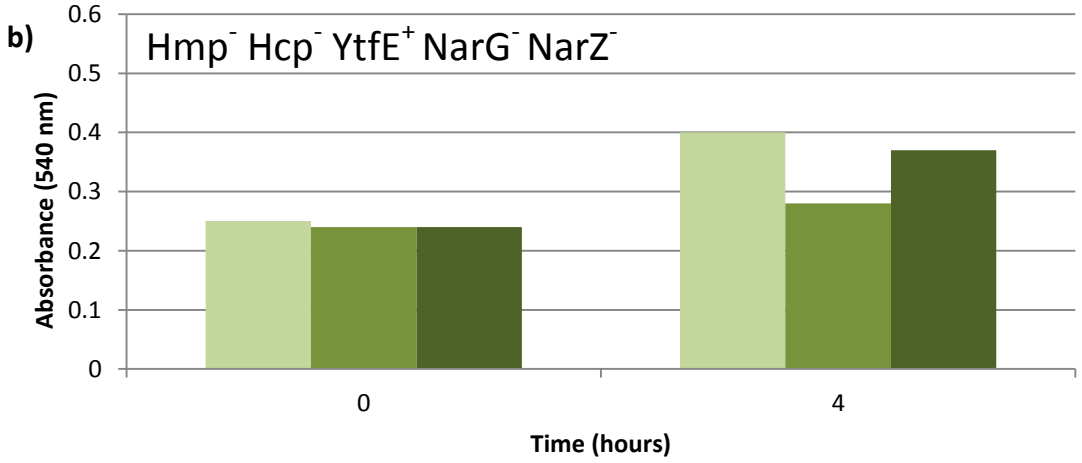
Sources of nitric oxide

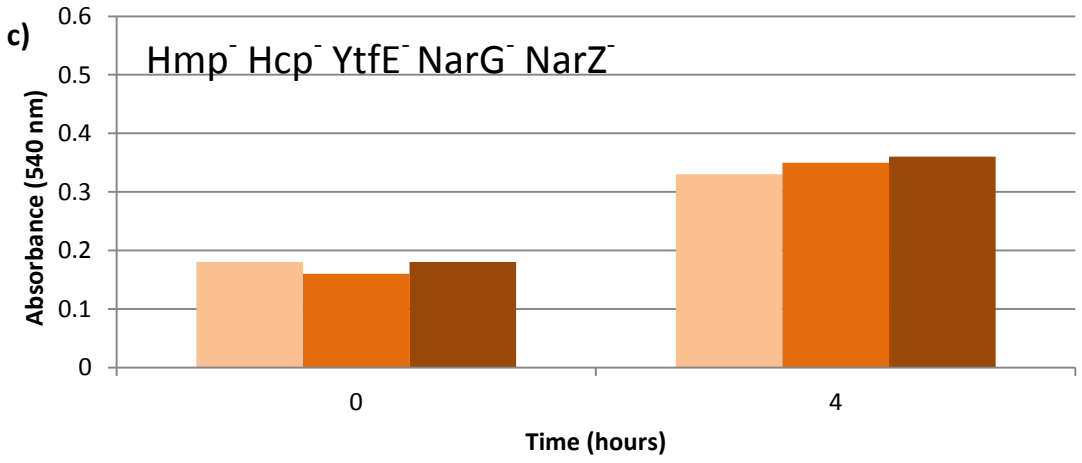
The major source of nitric oxide in *E. coli* has recently been confirmed as NarG (Vine, 2012), but as shown above YtfE is also capable of producing nitric oxide. The growth of strains defective in these sources of NO was investigated in the presence of nitrite and in the presence of nitrite with a NO scavenger, The *hcp* mutant, JCB5250, a strain defective in NarG and NarZ, JCB5270, and a strain defective in NarG, NarZ and YtfE, JCB5280, were grown in the absence of nitrite and PTIO, in the presence of 1.5 mM nitrite and in the presence of 1.5 mM nitrite and 1.5 mM PTIO. As previously seen the *hcp* mutant strain, JCB5250, grew well in the absence of nitrite and was inhibited in the presence of nitrite. PTIO prevented the growth inhibition seen with nitrite (Figure 3.10a). The $NarG^-$, $NarZ^-$ derivative, JCB5270, also grew well in the absence of nitrite, but again growth was inhibited in the presence of nitrite. In the presence of nitrite and PTIO growth inhibition was again relieved (Figure 3.10b). PTIO is again capable of mopping up the NO produced to restore growth, showing that nitric oxide is still produced in a strain deficient in the main source of nitric oxide, NarG. The $NarG^-$, $NarZ^-$, $YtfE^-$ strain, JCB5280, also grew well in the absence of nitrite. Unlike the isogenic $YtfE^+$ strain, this strain also grew well in the presence of nitrite and the presence of nitrite and PTIO (Figure 3.10c). This suggests that a *narG*, *narZ* and *ytfE* mutant is not sensitive to nitrosative stress,

Figure 3.10 Effect of NarG and NarZ mutations on the growth of strains sensitive to nitrosative stress in the presence of nitrite and PTIO.

The *hcp* mutant strain, JCB5250 (*hmp*, *hcp*), a NarG$^-$, NarZ$^-$ derivative, JCB5270 (*hmp*, *hcp*, *narG*, *narZ*) and a NarG$^-$, NarZ$^-$, YtfE$^-$ derivative, JCB5280 (*hmp*, *hcp*, *narG*, *narZ*, *ytfE)* were grown in the absence of nitrite (palest bar), in the presence of 1.5 mM nitrite (mid-pale bar) and in the presence of 1.5 mM nitrite and 1.5 mM PTIO. a) JCB5250 (*hmp*, *hcp*), b) JCB5270 (*hmp*, *hcp*, *narG*, *narZ*) c) JCB5280 (*hmp*, *hcp*, *narG*, *narZ*, *ytfE).* The x-axis shows the time in hours following addition of nitrite. This is representative data so no error bars are present.


a)
Hmp- Hcp- YtfE+ NarG+ NarZ+
Absorbance (540 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
Time (hours)


b)
Hmp- Hcp- YtfE+ NarG- NarZ-
Absorbance (540 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
Time (hours)


c)
Hmp- Hcp- YtfE- NarG- NarZ-
Absorbance (540 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
Time (hours)

possibly due to less NO being produced as all major sources of nitric oxide have been eliminated.

Confirmation that lack of a functional *hcp* gene causes sensitivity to nitrosative stress

Loss of a functional *hcp* gene causes growth inhibition in the presence of nitrosative stress, so complementation with a functional *hcp* gene from a plasmid should restore growth. The *hcp* mutant, discussed previously, JCB5250, was grown in the presence and absence of 0.5 mM nitrite, with and without pACYC184 Hcp, containing a functional *hcp* gene. In the absence of pACYC184 Hcp, the *hcp* mutant grew well in the absence of nitrosative stress, but growth was inhibited in the presence of nitrite as previously seen. In the presence of pACYC184 Hcp, the *hcp* mutant, now effectively Hcp^{+}, grew well in the absence and presence of nitrite (Figure 3.11). Growth was improved even in the absence of nitrosative stress by the presence of a functional *hcp* gene. Addition of a functional *hcp* gene can complement the phenotype seen in an *hcp* mutant both in the presence and absence of nitrosative stress.

The effect of *hcp* and *ytfE* mutations on sensitivity to NO

In the strains discussed above that are deficient in all known NO reductases, it appears to be an *hcp* mutation that makes them sensitive to nitrosative stress. Claire Vine (2011) reported that a further mutation in *ytfE* relieves inhibition of growth in the presence of nitrosative stress. To investigate whether a single mutation in either *hcp* or *ytfE* made cells more sensitive to nitrosative stress, the parent strain, RK4353, an *hcp* mutant, JCB4999, a *ytfE* mutant, RK4353 *ΔytfE* and an *hcp ytfE* double mutant, JCB5228, were grown anaerobically in the

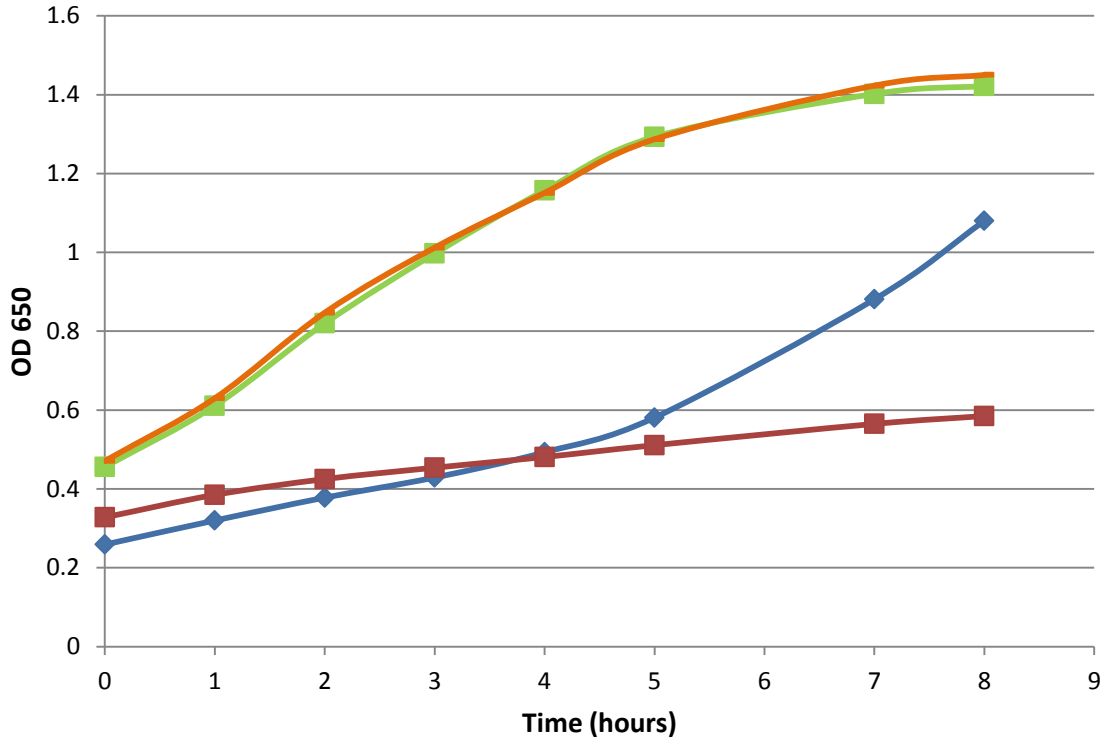
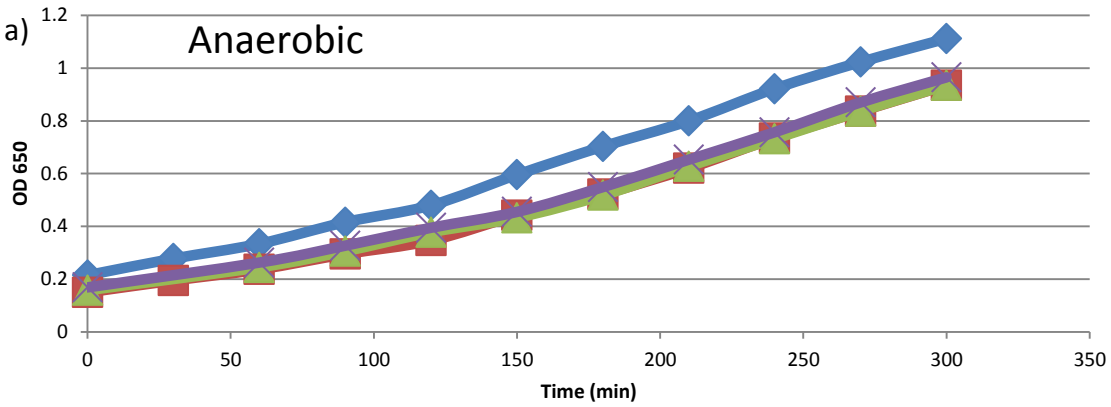


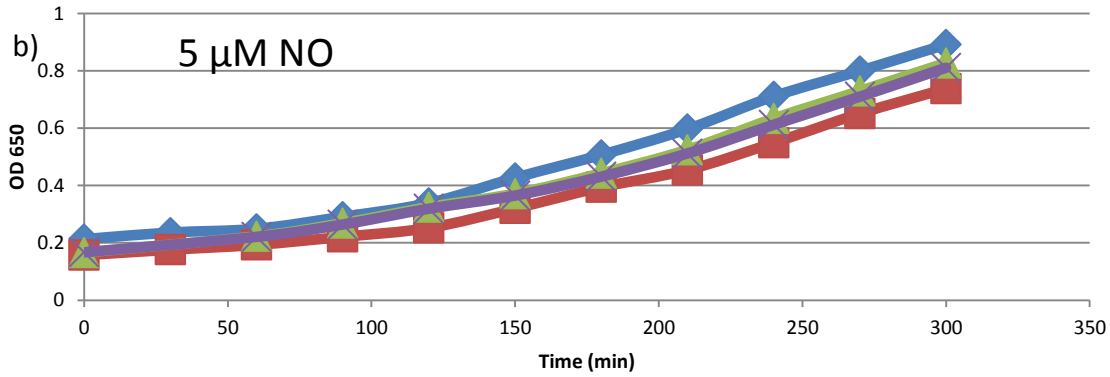
Figure 3.11 Complementation of the *hcp* mutant phenotype by expression of functional plasmid-borne genes encoding Hcp-Hcr.

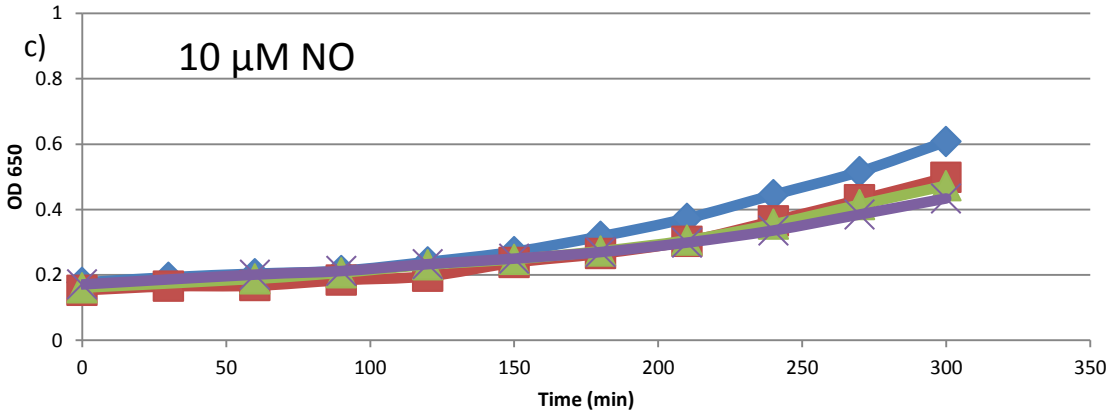
The *hcp* mutant, JCB5250, was grown anaerobically in the absence of a plasmid, without nitrite (Blue line) and with 0.5 mM nitrite (Red line), in the presence of a plasmid with Hcp in the absence of nitrite (Green line) and in the presence of a Hcp plasmid in the presence of nitrite (Orange line). This complementation was repeated with two independent isolates containing Hcp. The x-axis shows the time in hours following the addition of nitrite.

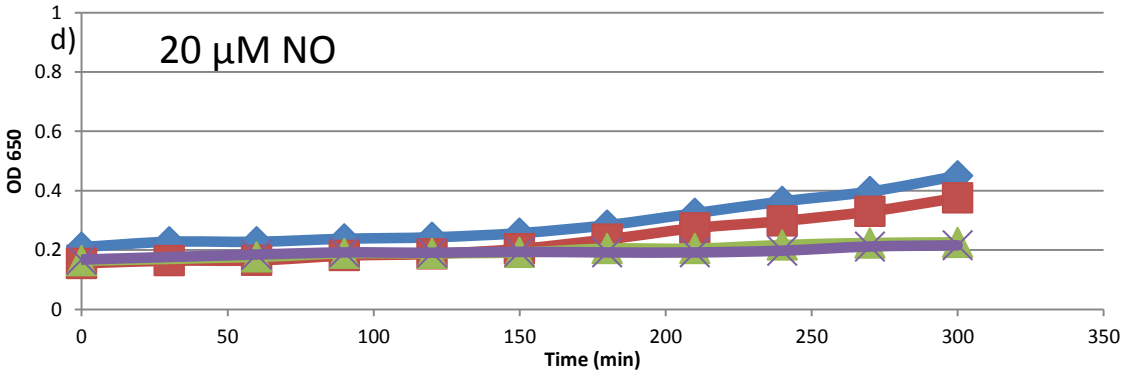
Figure 3.12 The effect of *hcp* and *ytfE* mutations on sensitivity to NO.

RK4353 (Blue line), an hcp mutant, JCB4999 (Green line), a *ytfE* mutant, RK4353 *ΔytfE* (Red line) and an *hcp ytfE* mutant, JCB5228 (Purple line), were grown anaerobically in the absence of NO or in the presence of 5, 10 or 20 μM NO. The x-axis is the time in minutes following the initial pulse of nitric oxide.


a)
Anaerobic
OD 650
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
300
350
Time (min)


b)
5 µM NO
OD 650
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
300
350
Time (min)


c)
10 µM NO
OD 650
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
300
350
Time (min)


d)
20 µM NO
OD 650
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
300
350
Time (min)

absence of NO or in the presence of 5, 10 or 20 μM NO. In the absence of NO all of the strains grew well (Figure 3.12a). In the presence of 5 and 10 μM NO growth of the *hcp* mutant, JCB4999, the *ytfE* mutant, RKK4353 *ΔytfE* and the *hcp, ytfE* mutant, JCB5228, was slightly inhibited compared to growth of the parent; however there was no difference in growth between the three strains (Figure 3.12b and 3.12c). Growth of the *hcp* mutant, JCB4999, the *ytfE* mutant, RK4353 *ΔytfE* and the *hcp, ytfE* mutant, JCB5228, was also inhibited in the presence of 20 μM NO, but the *hcp* mutant, JCB4999 and the *hcp ytfE* double mutant, JCB5228, were more sensitive than the *ytfE* mutant, RK4353 *ΔytfE,* compared to the parent strain (Figure 3.12d). These strains deficient in Hcp are more sensitive to high concentrations of NO under anaerobic conditions.

An assay for detecting the levels of nitric oxide in the cytoplasm

The promoters for *hcp* and *hmp* and are regulated by NsrR, which is a nitric-oxide sensing transcription factor that represses both of these promoters by binding to the promoter region and blocking RNA polymerase from transcribing the gene. When nitrosative stress is extreme, signalled by nitrate, nitrite and NO, NsrR binds NO. This prevents NsrR from binding to the promoter region, relieving repression and allowing transcription of the gene. Fusion of one of these promoters to the *lacZ* gene allows the relief of repression, due to NsrR binding to NO, to be monitored.

Effect of nitrite and nitrate during growth on transcription at the *hcp* promoter

To investigate the response of the *hcp* promoter to the presence of nitrite or nitrate during growth, plasmid phcp383-0, which contains the *hcp* promoter

fused to *lacZ* in pRW50, was transformed into the *E. coli* parent strain used in these experiments, JCB5232. This strain is a *nir*, *nor* and *nrf* mutant, so it is both *hmp*$^+$ and *hcp*$^+$. Purified transformants were grown anaerobically in the presence or absence of nitrite or nitrate; they were then lysed and assayed for β-galactosidase activity. The β-galactosidase activity due to transcription of *lacZ* from the *hcp* promoter was low in the absence of nitrate or nitrite. The activity had doubled within 2 hours during growth in the presence of nitrite, but not in the presence of nitrate (Figure 3.13a).

Induction of expression from the *hcp* promoter was not seen within two hours in the presence of nitrate. It was investigated whether the *hcp* promoter was induced by growth in the presence of nitrate over a longer period of time, in the same way as described previously. Induction was seen from 3 to 6 hours following addition of nitrate (Figure 3.13b). Nitrite is a more immediate source of nitric oxide than nitrate. This is because nitrate must first be reduced to nitrite, then further reduced to nitric oxide. In the presence of nitrate, nitrite reduction to nitric oxide is inhibited.

Effect of nitrite and nitrate during growth on transcription at the *hmp* promoter

As the *hcp* promoter is regulated by NsrR, it was investigated whether the same results were observed for another NsrR regulated promoter, *phmp*. To investigate the response of the *hmp* promoter to the presence of nitrite or nitrate during growth, pSP01, which contains the *hmp* promoter fused to *lacZ* in pRW50 was transformed into the parent strain, JCB5232. The cells were grown

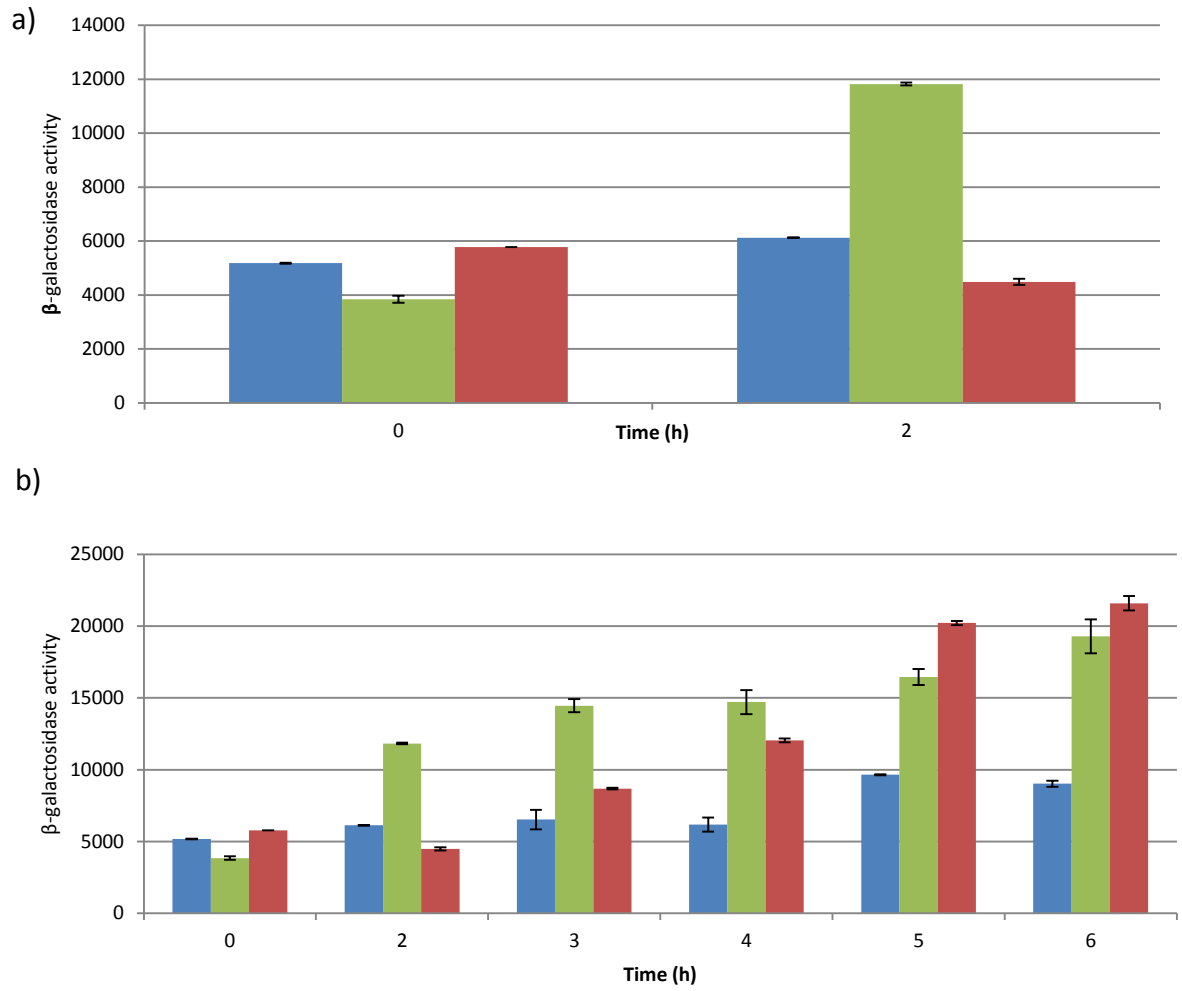


Figure 3.13 Effect of nitrate and nitrite, during growth, on transcription from the *hcp* promoter.

The parent strain, JCB5232 (defective in *nir*, *nrf* and *norVW* genes), with the *hcp:lacZ* fusion plasmid was grown anaerobically in the absence of nitrite and nitrate (blue bars), with 2.5 mM nitrite (green bars) and with 5 mM nitrate (red bars). Units of β-galactosidase activity are nmol ONPG hydrolysed min^{-1} (mg dry weight)$^{-1}$ in this and subsequent figures. The x-axis shows the time in hours following the addition of nitrate or nitrite. Error bars in this and subsequent figures show the standard error of three repeats.

anaerobically in the presence or absence of nitrite or nitrate; they were then lysed and assayed for β-galactosidase activity. The β-galactosidase activity due to transcription of *lacZ* from the *hmp* promoter was low in the absence of nitrite or nitrate. The activity had increased within 2 hours when nitrite was present during growth, as seen with the *hcp* promoter, but not when nitrate was present during growth. However, an increase in β-galactosidase activity was seen from 3 to 6 hours following addition of nitrate (Figure 3.14). This is consistent with the presence of nitrate inhibiting the reduction of nitrite to nitric oxide. Therefore, any nitrate must first be reduced to nitrite before the nitrite can be further reduced to nitric oxide.

Response of *hcp* and *hmp* promoters to nitric oxide

NsrR is an NO sensing transcription factor. Nitrate is reduced to nitrite, which is then converted to NO allowing the presence of nitrate or nitrite during growth to affect expression of the *hcp* and *hmp* promoters. The effects of repeated additions of 5 μM NO during growth were investigated. To investigate the response of the *hcp* and *hmp* promoters to the presence of NO during growth, phcp383-0 and pSP01, the *hcp* and *hmp* promoters fused to *lacZ* in pRW50 were transformed into the parent strain, JCB5232, deficient in *nirB*, *norVW* and *nrfA*. The cells were grown anaerobically either in the absence of NO or with sequential additions of NO every 30 minutes; they were then lysed and assayed for β-galactosidase activity.

The β-galactosidase activity due to transcription of *lacZ* from the *hcp* and *hmp* promoters was low in the absence of NO during growth. No significant increase in activity was seen within 2 hours following the first addition of NO for either

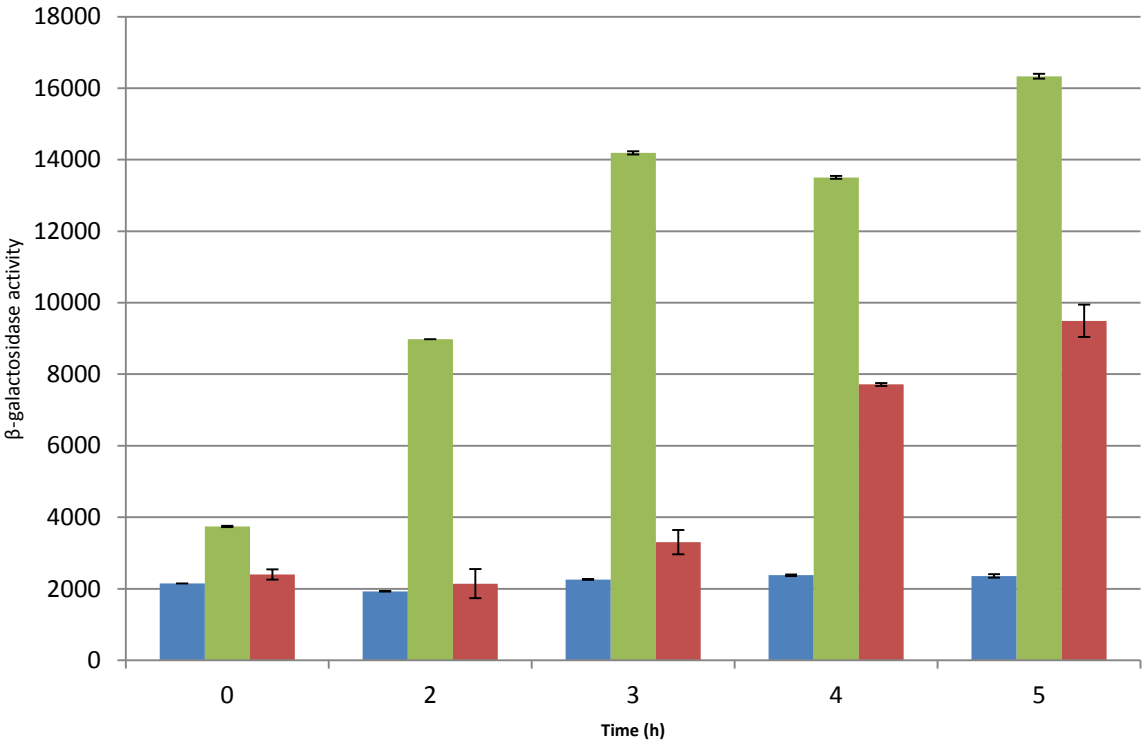


Figure 3.14 Effect of nitrite and nitrate, during growth, on expression of the *hmp* promoter.

The parent strain, JCB 5232, with the *hmp:lacZ* fusion plasmid was grown anaerobically in the absence of nitrite and nitrate (blue bars), with 2.5 mM nitrite (green bars) and with 5 mM nitrate (red bars). The x-axis shows the time in hours following the addition of nitrate or nitrite.

promoter. However, activity did increase between 3 and 6 hours following addition of NO. This increase in activity with NO was not as dramatic as that seen with nitrite or even nitrate (Figure 3.15). Nitric oxide appears to be less effective than nitrate and especially nitrite as a source of nitrosative stress. As nitric oxide is a small, uncharged molecule it is assumed that it will equilibrate across the membrane when added externally to the cells. However the delayed and ineffective response seen with NO suggests that it cannot cross the membrane very easily. It therefore cannot accumulate in the cytoplasm to relieve NsrR repression. Nitrate and nitrite are capable of being transported across the cytoplasmic membrane into the cytoplasm, where they are then reduced to nitric oxide. Nitric oxide cannot cross this membrane as easily, causing a delay in the response to nitric oxide as a source of stress. It is possible that Hcp itself is in the membrane acting as a barrier to NO.

The effect of an *hmp* mutation on expression of the *hmp* and *hcp* promoters

It was concluded from previous experiments that an *hmp* mutation had no effect on growth in the presence of nitrosative stress. To investigate the effect of an *hmp* mutation on the expression of the *hmp* and *hcp* promoters, the same plasmids as above were transformed into the *hmp* mutant, JCB5210. The cells were grown in the absence of nitrosative stress, in the presence of 5 mM nitrate or in the presence of repeated additions 1 μM NO. Cells were then lysed and assayed for β-galactosidase activity. The β-galactosidase activity due to transcription of *lacZ* from the *hcp* and *hmp* promoters was again low in the absence of nitrosative stress. Activity increased 2-fold in the presence of nitrate, for both promoters, by 3 hours following the addition of stress. Activity in the

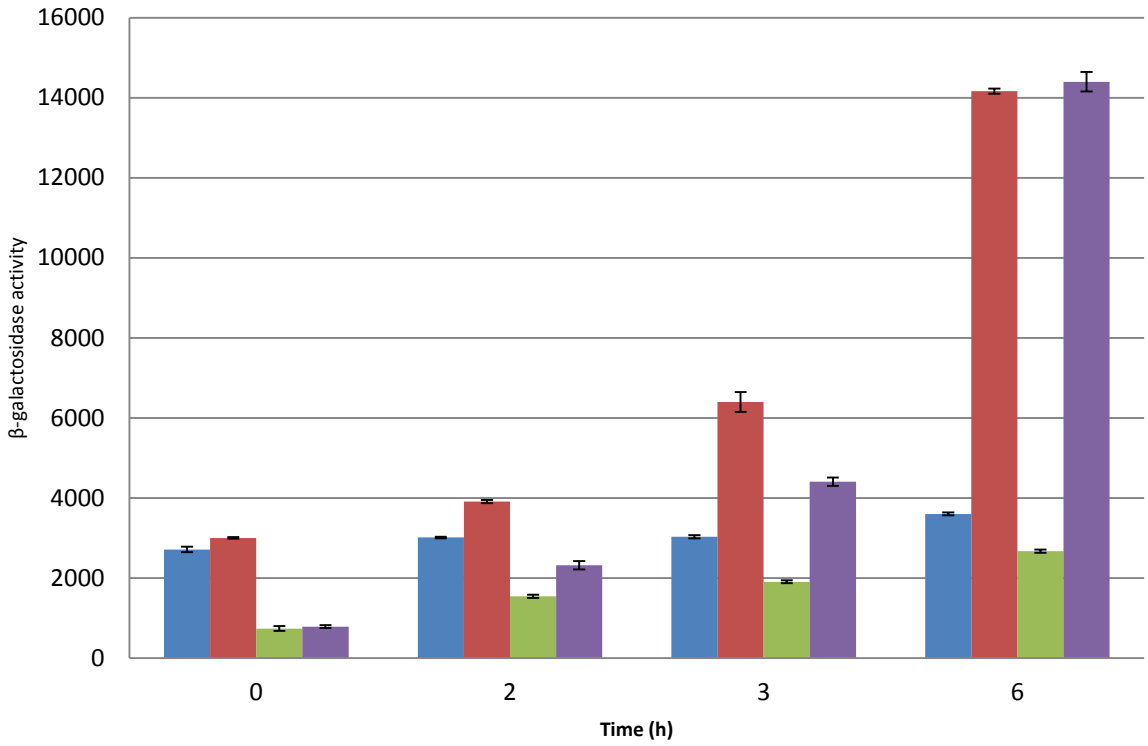


Figure 3.15 Effect of NO, during growth, on expression of the *hcp* and *hmp* promoters.

The parent strain, JCB5232, with the *hcp:lacZ* fusion plasmid was grown anaerobically in the absence of NO (blue bars) and with 5 μM NO (red bars), with the *hmp:lacZ* in the absence of NO (green bars) and with 5 μM NO (purple bars). The x-axis shows the time in hours following the first addition of nitric oxide.

presence of nitric oxide had also increased by 3 hours following the addition of stress, for both promoters, but to a lesser extent than in the presence of nitrate (Figure 3.16). Nitric oxide was not as effective as nitrate in inducing expression of the promoters. This was previously seen with the parent strain, JCB5232, showing that an *hmp* mutation had no effect on expression from the *hmp* or *hcp* promoters. Hmp therefore appears to be unimportant for the reduction of NO during anaerobic growth in the presence of nitrosative stress.

The effect of an *hcp* mutation on expression of the *hmp* and *hcp* promoters

Hcp appears to be essential for anaerobic growth in the presence of nitrosative stress. To investigate the effect of an *hcp* mutation on expression of the *hmp* and *hcp* promoters, the same plasmids as previously described were transformed into the *hcp* mutant, JCB5250 (*hmp*, *hcp*), and the cells were grown as previously described, lysed and assayed for β-galactosidase activity. The β-galactosidase activity due to transcription of *lacZ* from the *hcp* and *hmp* promoters was high under all conditions for both promoters (Figure 3.17), as high as previously seen when β-galactosidase activity increased in the presence of nitrate and nitrite. Even in the absence of nitrosative stress NsrR appears to be inactive in an *hcp* mutant, and unable to repress transcription at the *hmp* and *hcp* promoters.

The effect of a *ytfE* mutation on expression of the *hmp* and *hcp* promoters

A *ytfE* mutation in an *hcp* mutant strain is capable of restoring growth inhibition. To investigate the effect of an *ytfE* mutation on expression of the *hmp* and *hcp*

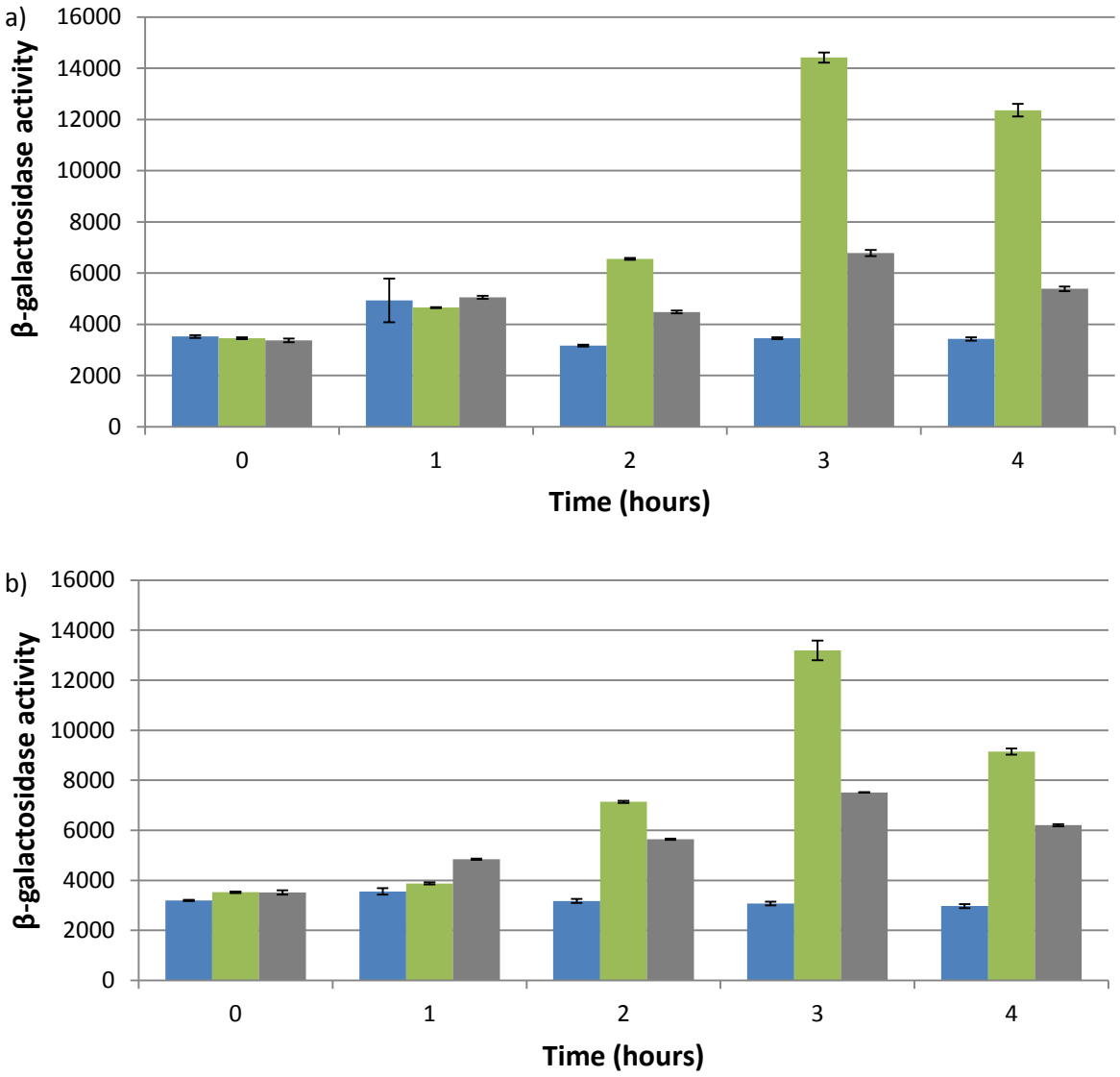


Figure 3.16 Effect of an *hmp* mutation on expression from the *hmp* and *hcp* promoters.

The *hmp* mutant strain, JCB5210, with the *hcp:lacZ* fusion plasmid (a) and with the *hmp:lacZ* plasmid (b) were grown anaerobically in the absence of nitrosative stress (blue bars), in the presence of 5 mM nitrate (green bars) and in the presence of 1 μM NO (grey bars). The x-axis shows the time in hours following the addition of nitrate or nitric oxide.

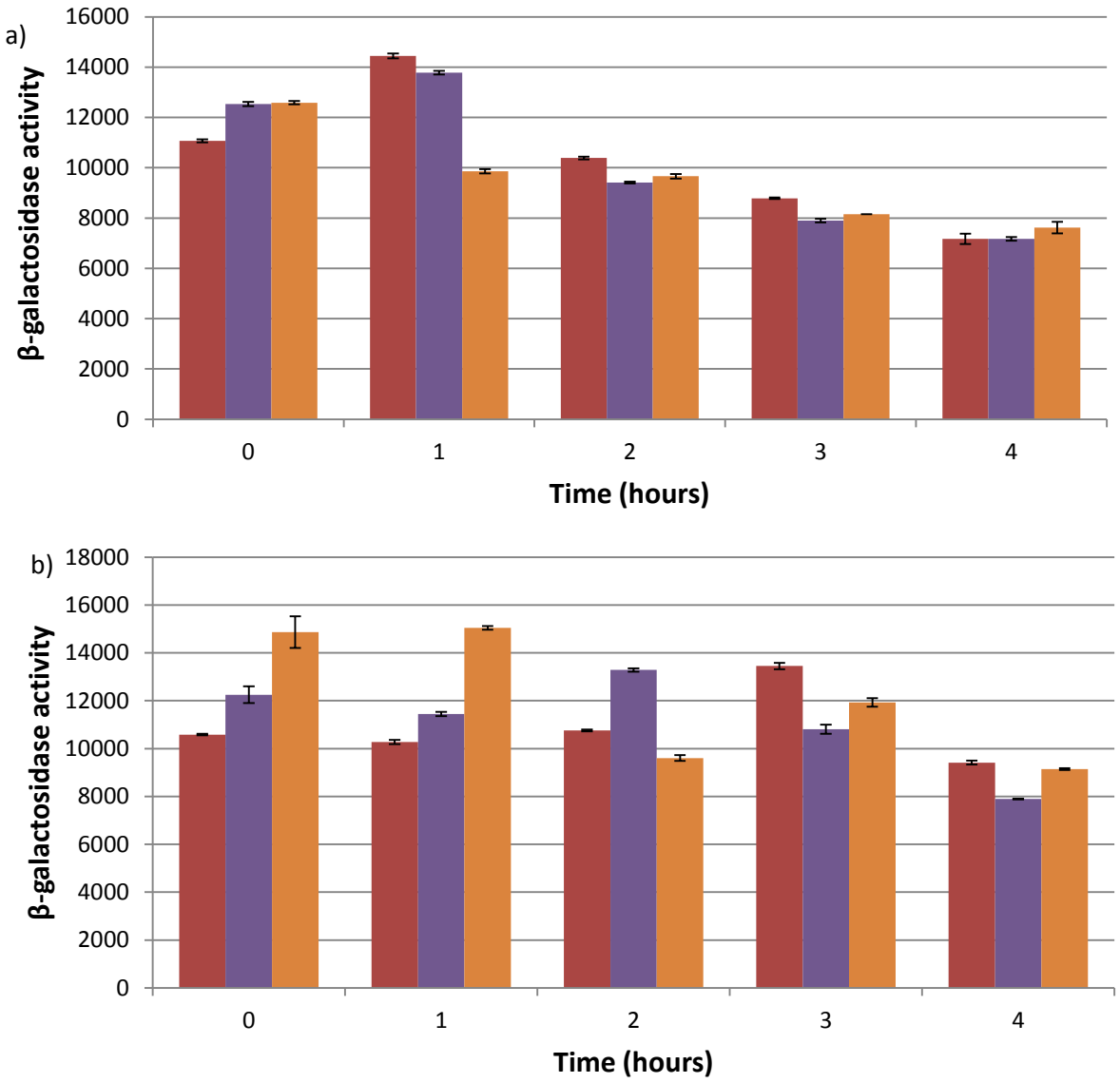


Figure 3.17 Effect of an *hcp* mutation on expression from the *hmp* and *hcp* promoters.

The *hcp* mutant strain, JCB5250, with the *hcp:lacZ* fusion plasmid (a) and with the *hmp:lacZ* plasmid (b) were grown anaerobically in the absence of nitrosative stress (red bars), in the presence of 5 mM nitrate (purple bars) and in the presence of 1 μM NO (orange bars). The x-axis shows the time in hours following the addition of nitrate or nitric oxide.

promoters, the same plasmids as before were transformed into the *ytfE* mutant, JCB5260 (*hmp*, *hcp*, *ytfE*), and the cells were grown and assayed as before.

The β-galactosidase activity due to transcription of *lacZ* from the *hcp* and *hmp* promoters was still partially elevated in all conditions for both promoters (Figure 3.18), although not to the extent as in the isogenic *ytfE*$^+$ strain, JCB6250. These results show that a *ytfE* mutation in a *hcp* mutant background can partially reduce expression of the *hmp* and *hcp* promoters, possibly due to partial reactivation of NsrR, allowing partial repression of the genes.

The effect of a *ytfE* mutation in a Hcp$^+$ background on expression of the *hmp* and *hcp* promoters

To investigate if a *ytfE* deletion has any effect in a Hcp$^+$ background on expression of the *hmp* and *hcp* promoters the same plasmids as before were transformed into the *ytfE* mutant strain, JCB5257 (*hmp*, *ytfE*) and the cells were grown and assayed as before. The β-galactosidase activity due to transcription of *lacZ* from the *hcp* and *hmp* promoters was low in the absence of nitrosative stress, for both promoters. Activity at both promoters increased 2-fold by 2 hours following the addition of nitrate. Activity at both promoters also increased by 2 hours following the addition of nitric oxide, but to a lesser extent than in the presence of nitrate (Figure 3.19) as previously seen in the isogenic ytfE$^+$ strain, JCB5210. A *ytfE* mutation has no effect in a Hcp$^+$ strain on expression from the *hmp* and *hcp* promoters.

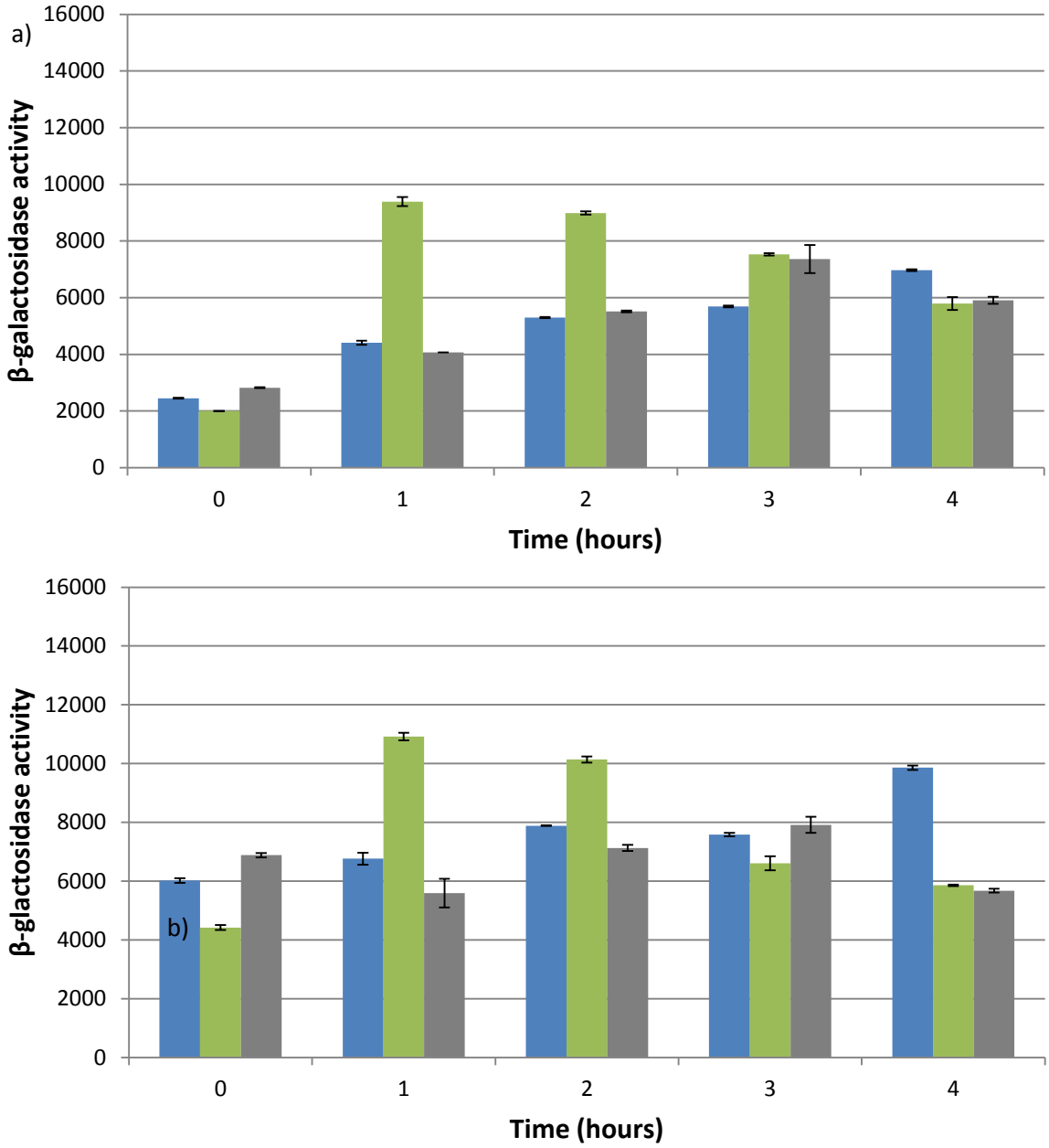


Figure 3.18 Effect of an *ytfE* mutation on expression from the *hmp* and *hcp* promoters.

The *ytfE* mutant strain, JCB5260, with the *hcp:lacZ* fusion plasmid (a) and with the *hmp:lacZ* plasmid (b) were grown anaerobically in the absence of nitrosative stress (blue bars), in the presence of 5 mM nitrate (green bars) and in the presence of 1 µM NO (grey bars). The x-axis shows the time in hours following the addition of nitrate or nitric oxide.

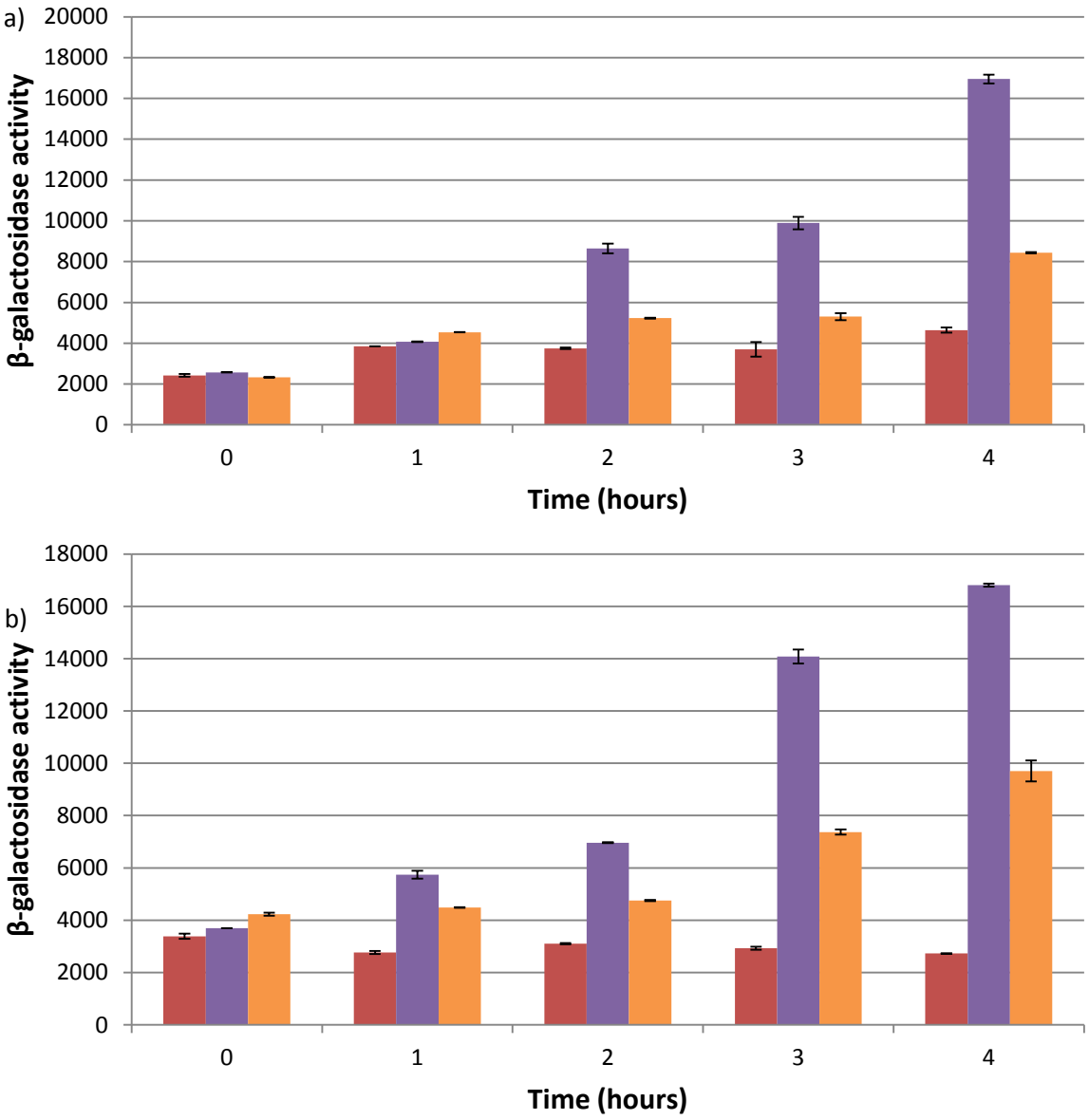


Figure 3.19 Effect of an *ytfE* mutation, in an Hcp^{+} background, on expression from the *hmp* and *hcp* promoters.

The *ytfE* mutant strain, JCB5257, with the *hcp:lacZ* fusion plasmid (a) and with the *hmp:lacZ* plasmid (b) were grown anaerobically in the absence of nitrosative stress (red bars), in the presence of 5 mM nitrate (purple bars) and in the presence of 1 μM NO (orange bars). The x-axis shows the time in hours following the addition of nitrate or nitric oxide.

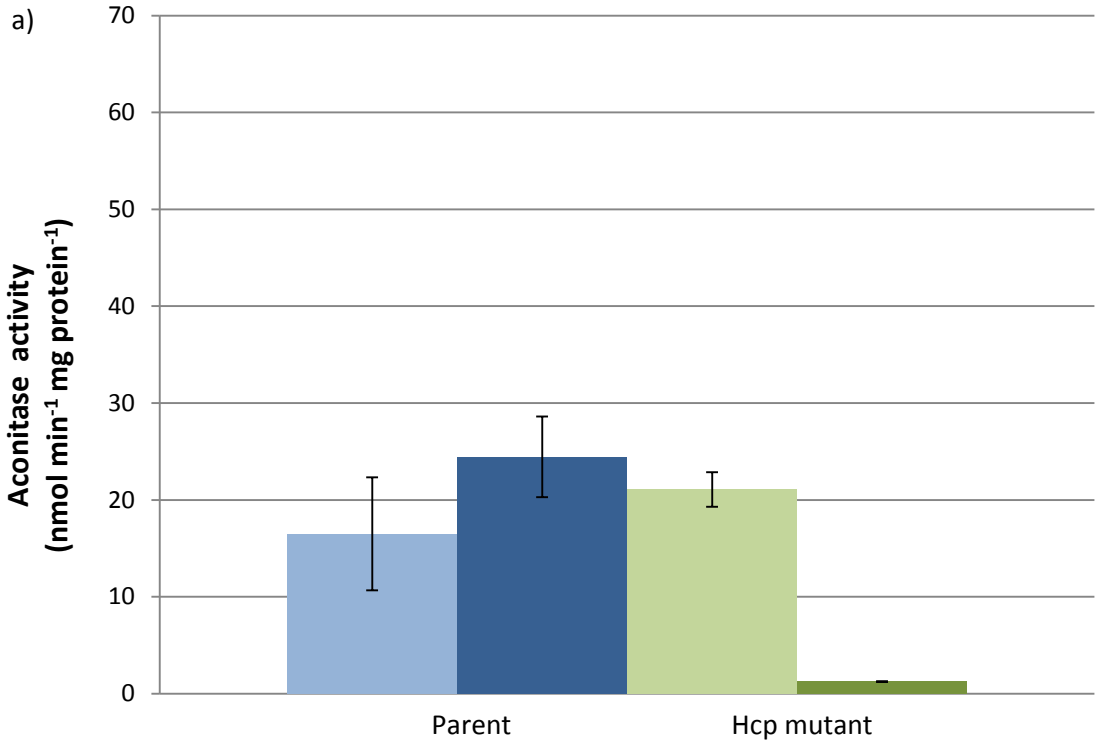
Iron-sulphur proteins damaged by nitrosative stress

Proteins containing iron-sulphur clusters are damaged in the presence of nitrosative stress. In enzymes such as aconitase and fumarase, that contain an iron-sulphur cluster, the cluster becomes nitrosylated by nitric oxide causing inactivation of the enzyme. An *hcp* mutant shows inhibited growth in the presence of nitrosative stress and high β-galactosidase activity due to transcription of *lacZ* from the *hcp* and *hmp* promoters in the presence of nitrosative stress. The effect of nitrosative stress on the activity of these two enzymes in strains sensitive to nitrosative stress was investigated. The parent strain for these experiments, JCB5210, and the *hcp* mutant, JCB5250, were grown in the presence and absence of 5 mM nitrate, cells were then harvested, washed, and broken in the French press. After high speed centrifugation the soluble extract was assayed for both aconitase and fumarase activity. Aconitase activity in the parent strain, JCB5210, was not lower in the presence of nitrosative stress compared to the absence of stress. In contrast, aconitase activity in the *hcp* mutant, JCB5250, was lower after growth in the presence of nitrosative stress compared to in the absence of stress (Figure 3.20a). An *hcp* mutation resulted in almost completely inactive aconitase in the presence of nitrosative stress, suggesting that Hcp either protects aconitase from damage, or repairs aconitase damaged by nitrosative stress. Fumarase in the parent strain, JCB5210, was also active in the presence of nitrosative stress but again activity in the *hcp* mutant, JCB250, was decreased in the presence of nitrosative stress (Figure 3.20b). An *hcp* mutation again resulted in inactive enzyme in the presence of stress.

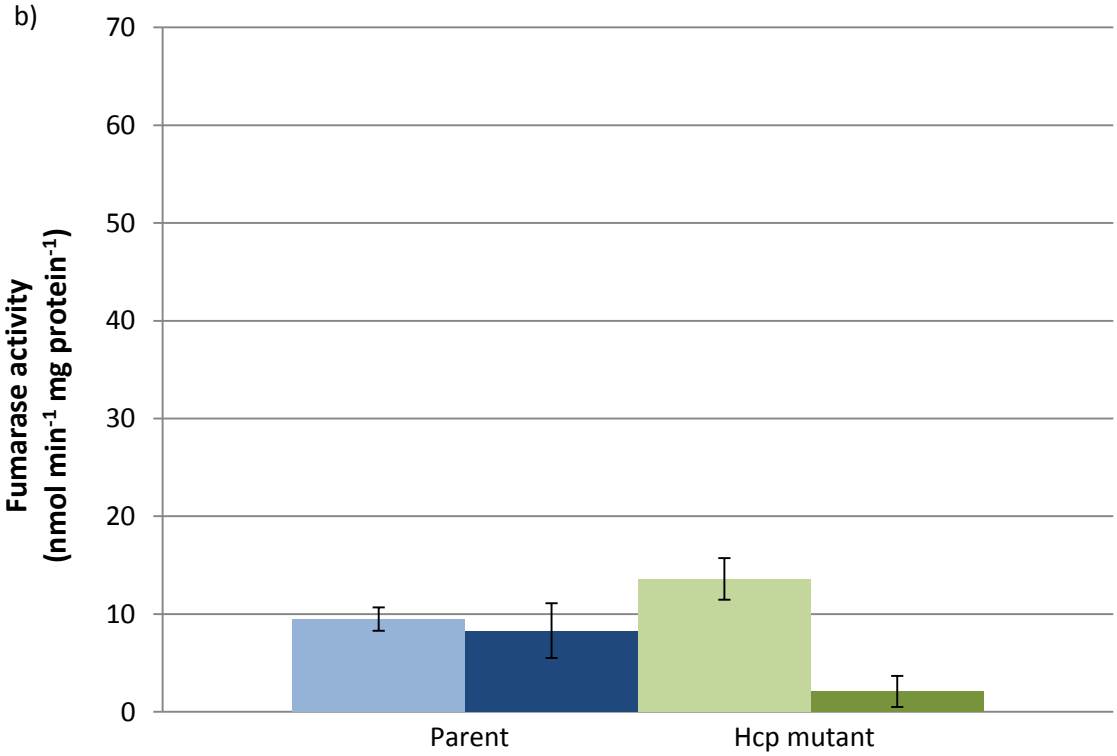
Figure 3.20 Effect of an *hcp* mutation on activity of iron-sulphur cluster enzymes, aconitase and fumarase.

The parent strain, JCB5210 (blue bars), and the *hcp* mutant, JCB5250 (green bars), were grown in the absence of nitrate (pale bars) and in the presence of 5 mM nitrate (dark bars) and assayed for aconitase activity (a) and fumarase activity (b). Activity has been corrected for protein concentration. Error bars show the standard error of two independent replicates for aconitase and four independent replicates for fumarase.

a)


70
60
50
40
30
20
10
0
Aconitase activity
(nmol min-1 mg protein-1)
Parent
Hcp mutant

b)


70
60
50
40
30
20
10
0
Fumarase activity
(nmol min-1 mg protein-1)
Parent
Hcp mutant

Conclusions

Previous work by Claire Vine (2011) has shown that a strain deficient in all known NO reductases, including Hmp, is not sensitive to nitrosative stress. The results of this present study are in agreement with previous work and suggest that an alternative NO reducing system(s) must be present. However, a strain deficient in Hcp shows severe inhibited growth in the presence of nitrosative stress, even in the presence of a known NO reductase, Hmp. This suggests that Hcp not Hmp is vital for resistance to nitrosative stress. This inhibited growth was suggested to be due to the build-up of a toxic product. To confirm that Hcp was responsible for the inhibited growth seen in the *hcp* mutant, a functional *hcp* gene was introduced into this strain. The addition of this functional gene restored growth in both the presence and absence of nitrosative stress.

In previous experiments the introduction of a *ytfE* mutation into a strain deficient in all known NO reductases and Hcp relieved inhibited growth. This suggested YtfE was responsible for producing the toxic product. This work has shown that the toxic product produced by YtfE is unlikely to be Fe^{2+}, as the addition of 2,2-dipyridyl did not relieve the growth inhibition. It has also shown that addition of ferrous sulphate can in fact restore the inhibited growth seen in an *hcp* mutant both in the presence and absence of nitrosative stress in a dose-dependent manner. This restoration of growth can even occur at a point where nitrosative damage will have already occurred. This suggests ferrous ions are required for repair of nitrosative damage. The addition of PTIO, a scavenger for NO, also relieved the inhibited growth. As removal of NO relieved growth inhibition, the toxic product produced by YtfE that causes the poor growth is likely to be NO itself. A strain deficient in the main source of NO, NarG

(and NarZ) still produced NO. However, when a further mutation in YtfE was introduced into this strain, no growth inhibition was observed. Furthermore, less NO is produced as all major enzyme sources of NO are inactivated, these findings demonstrate that the toxic product produced by YtfE is in fact NO.

An assay for detecting the level of NO in the cytoplasm was also used to identify which gene plays a vital role in the response to nitrosative stress. The presence of nitrosative stress increases transcription at the *hcp* promoter and the *hmp* promoter, due to NO inhibiting repression by NsrR. However, an *hmp* mutation has no effect on the transcription from these promoters. Hmp appears to be unimportant for the reduction of NO during anaerobic growth in the presence of nitrosative stress. When an *hcp* mutant background was used for the assay, NsrR appeared unable to repress transcription at either of the promoters. Again, confirming that Hcp, not Hmp plays a vital role in the nitrosative stress response. When a YtfE mutation was introduced NsrR repression of the promoters was partially restored. Possibly due to less NO being produced to inhibit NsrR repression.

Finally, the activities of two iron-sulphur cluster proteins, aconitase and fumarase were used to investigate the effect of nitrosative damage. In an *hcp* mutant, both enzymes had very low activities. Hcp is important for functional iron-sulphur cluster proteins. Hcp may either protect against or repair damage caused by nitrosative stress.

CHAPTER 4 – RESULTS

Removal of nitric oxide - Introduction

Strains deficient in all known NO reductases still show 50% of the NO reduction activity of the parent strain (Vine, 2011), suggesting that other systems remain to be characterized. Genes suspected to be involved in the reduction of nitric oxide can be mutated and the effect on the rate of NO reduction monitored using a nitric oxide sensitive electrode.

Kinetics of the rate of NO reduction

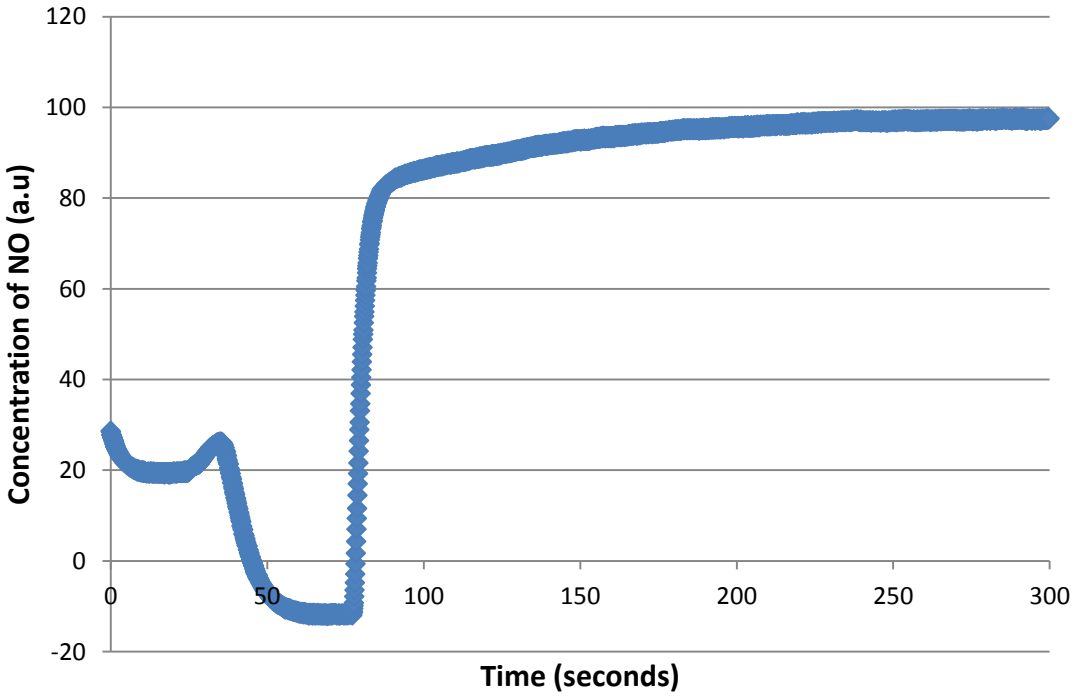
An assay for NO reduction using a Hansatech Oxytherm electrode was used to measure the rate of NO reduction in cells. Cells were grown in the presence or absence of nitrate or nitrite and the rates of NO reduction were measured upon addition of NO-saturated water.

To investigate the kinetics of the rate of NO reduction, various concentrations of NO-saturated water were added during the assay and the rate of NO reduction was measured. The kinetics of the parent strain, RK4353, were compared to that of a strain deficient in all characterised NO reductases, Nir, Nor, Nrf and Hmp, JCB5210. Strains were grown anaerobically in the presence of 20 mM nitrate, harvested and washed. Experimental reagents were added to the electrode chamber and the trace was left to stabilize until a steady-state zero concentration of oxygen was seen. NO was added (25-150 μM) and the initial rate of NO reduction was recorded (Figure 4.1). No significant decrease in the concentration of NO was detected in the absence of bacteria (Figure 4.1a).

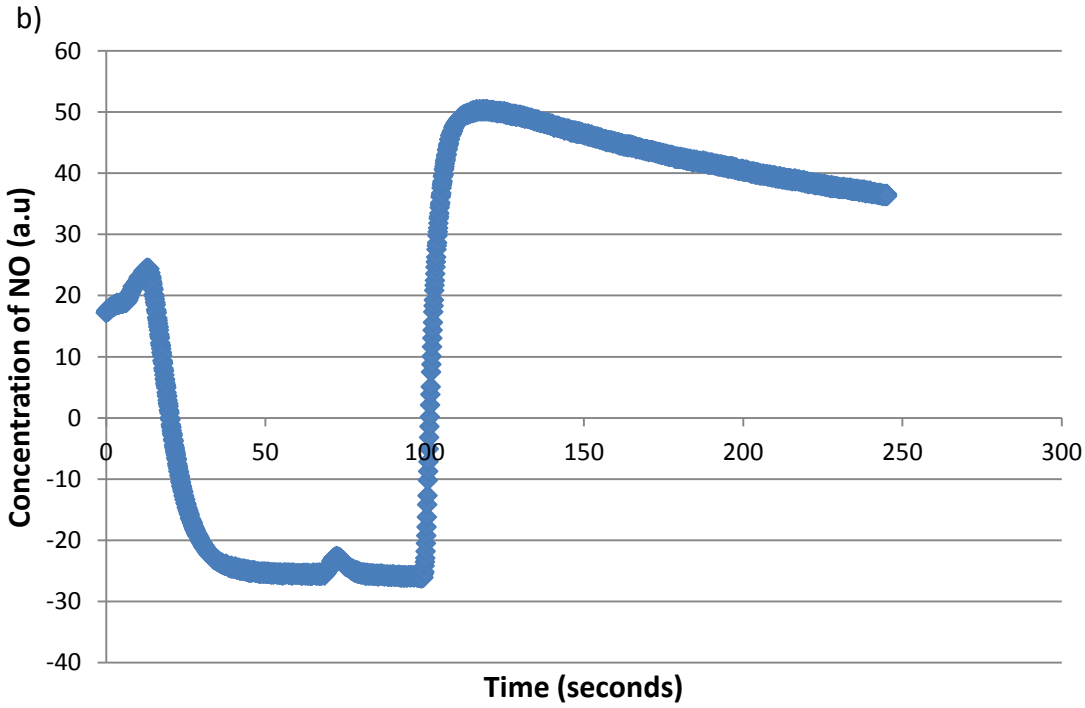
Figure 4.1 Representative raw data trace from the NO electrode

The control containing all the components required minus cells (a) and a representative trace containing cells that were grown, washed, harvested and assayed for NO reduction activity with 150 μM NO (b). The slope of the trace was given by the 'Rate measurement' function of the electrode and the rate was calculated using the equation as previously described. Each assay was repeated at least three times to achieve consistent rates for each condition.

a)


120
100
80
60
40
20
0
-20
0
50
100
150
200
250
300
Concentration of NO (a.u)
Time (seconds)

b)


60
50
40
30
20
10
0
-10
-20
-30
-40
0
50
100
150
200
250
300
Concentration of NO (a.u)
Time (seconds)

NO is added to the exterior of the cells in these experiments, and it is this extracellular NO that is measured by the electrode. Intracellular concentrations of NO may not vary directly in a linear fashion as incoming NO must diffuse through both polar and non-polar environments and, as NO is highly reactive, is likely to react with both extracellular and periplasmic components before it reaches the cytoplasm. Any values obtained for K_M and V_{max} are estimates of these enzymatic parameters. Had these estimates been generated using internally generated NO from nitrate or nitrate the values may have been different and possibly more accurate.

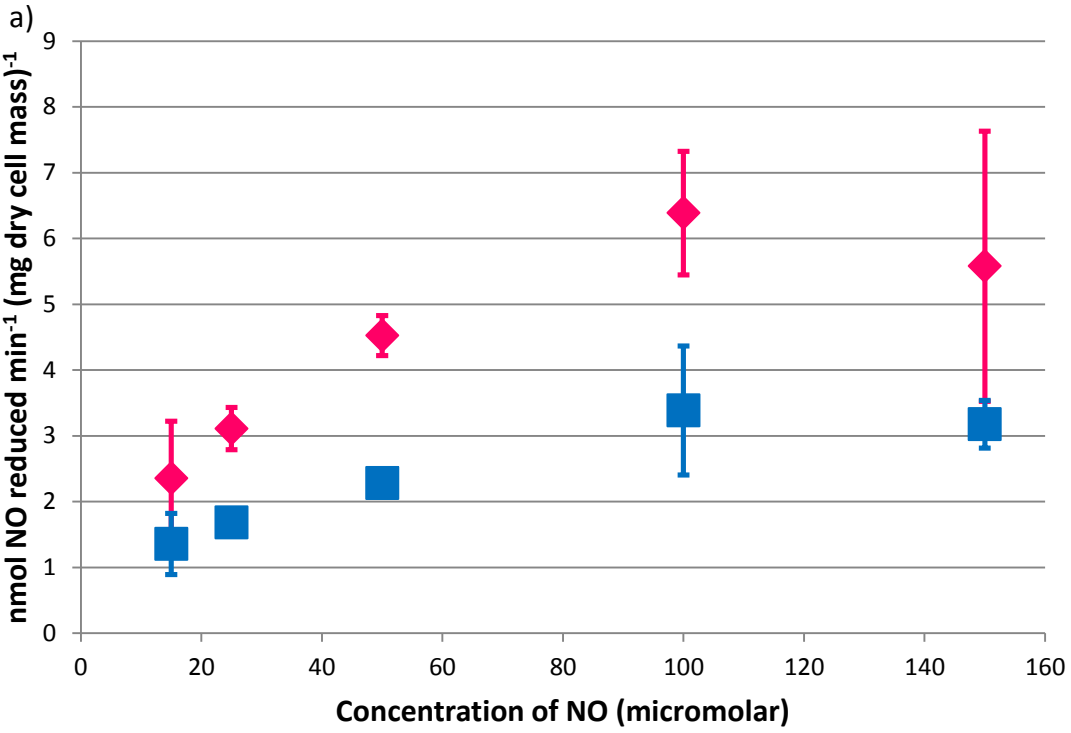
Both the parent strain and the NO reductase mutant strain appeared to reduce NO with Michaelis–Menten kinetics (Figure 4.2). The rates observed for the mutant strain, JCB5210, were half that seen for the parent (Figure 4.2a). However, upon addition of twice the amount of cells for the mutant the rates seen were similar so the kinetics of the two strains could be better compared (Figure 4.2b). For both strains, the rate of NO reduction was hyperbolically dependent upon the concentration of NO added so the data was combined to provide an estimate of $V_{max}^{app.}$ and $K_M^{app.}$. The data was analysed using the equation below:

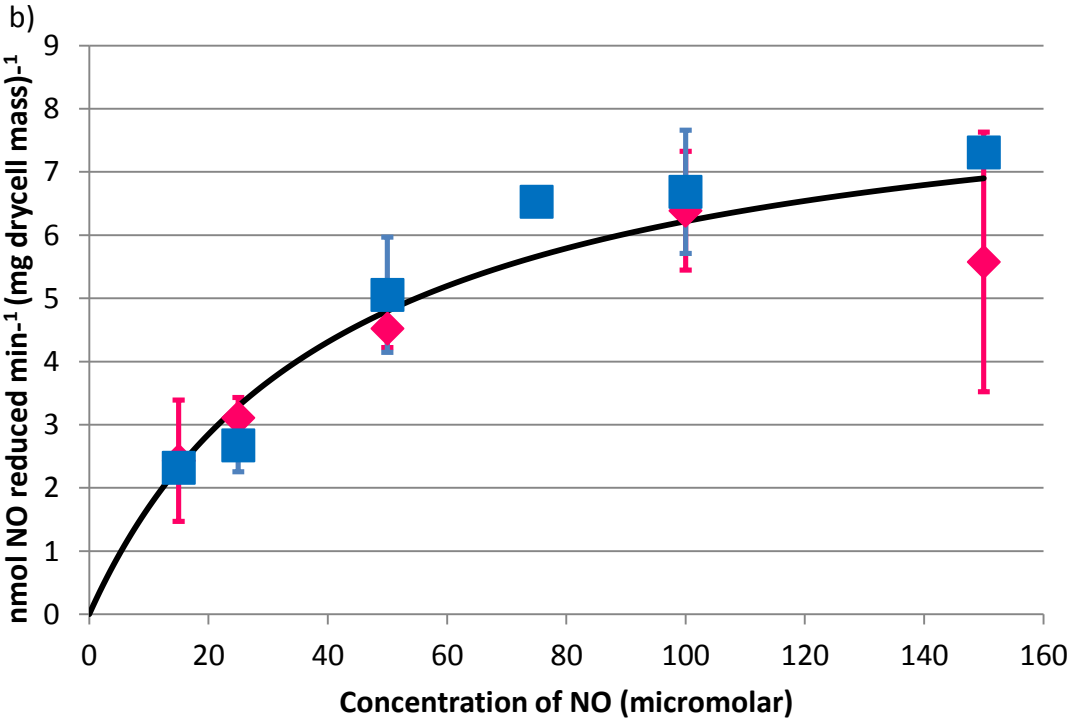
$$\text{Rate of NO Reduction} = \frac{V_{max}^{app.}[\text{extracellular NO}]}{K_M^{app.} + [\text{extracellular NO}]}$$

Estimates for the values of $V_{max}^{app.}$ and $K_M^{app.}$ were calculated using SigmaPlot, using a non-linear regression, global curve fitting programme and a single rectangular hyperbolic curve.

Figure 4.2 Kinetics of the rate of NO reduction in the parent strain and a strain deficient in all known NO reductases, JCB5210.

The parent strain RK4353 (pink symbols), and a strain deficient in all known reductases JCB5210 (blue symbols), were grown in the presence of nitrate, harvested and assayed for NO reduction activity. a) NO reduction activity with the same volume of cells for each strain b) NO reduction activity with twice as many cells of the mutant strain compared to the parent strain. The points are an average value of 3 repeats and the error bars show the standard deviation of the data. The data was analysed using the Michaelis-Menton equation. The black line shows the rectangular hyperbolic curve of the data. The values of the parameters $K_M^{app.}$ and V_{max}^{app} were generated using SigmaPlot and are given under the graph.


a)
nmol NO reduced min-1 (mg dry cell mass)-1
0
1
2
3
4
5
6
7
8
9
0
20
40
60
80
100
120
140
160
Concentration of NO (micromolar)


b)
nmol NO reduced min-1 (mg drycell mass)-1
0
1
2
3
4
5
6
7
8
9
0
20
40
60
80
100
120
140
160
Concentration of NO (micromolar)

A value for $V_{max}^{app.}$ of 8.8316 +/- 0.8174 nmol NO reduced min^{-1} (mg dry cell mass)$^{-1}$ and a value for $K_M^{app.}$ of 42.0084 +/- 10.4163 µM NO was calculated. The data was confirmed to show a high level of fit with an R^2 value of 0.9380, an adjusted R^2 value of 0.9318 and a standard error of the estimate of 0.5903.

One of the other points observed from the data was that the concentration of NO that appeared to give the highest and most consistent rates was 100 µM NO. The rates seen from 150 µM NO were similar to those seen for 100 µM most of the time but were sometimes lower, possibly because these very high concentrations caused excessive nitrosative damage as NO is highly reactive.

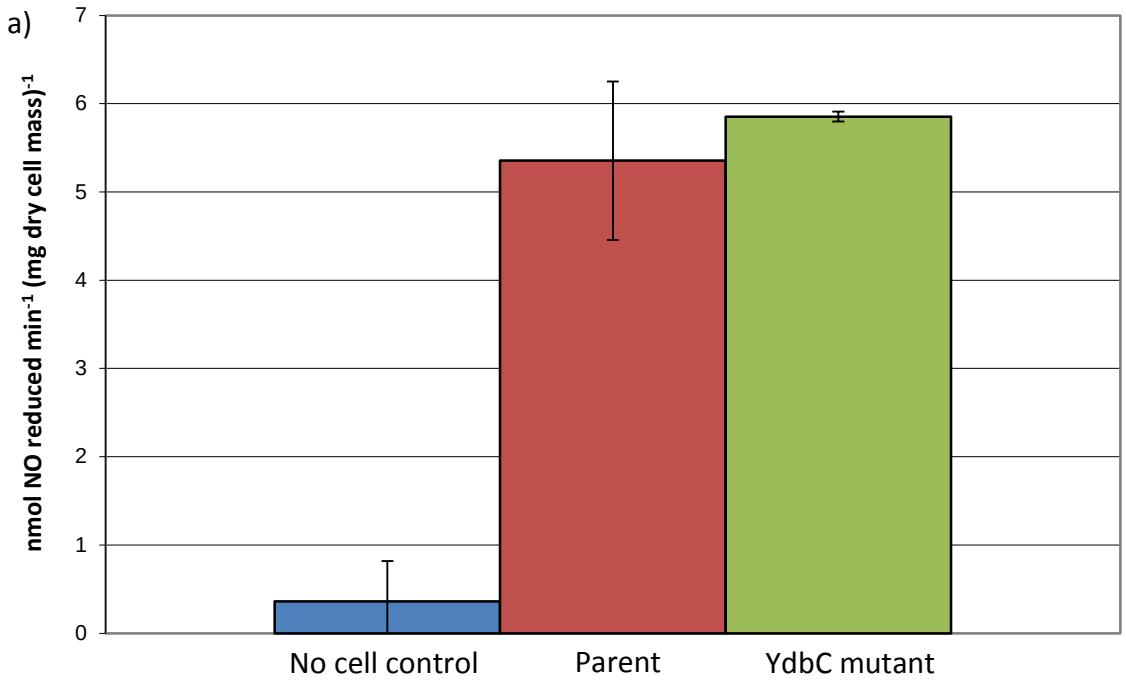
Evidence that YdbC is not an NO reductase

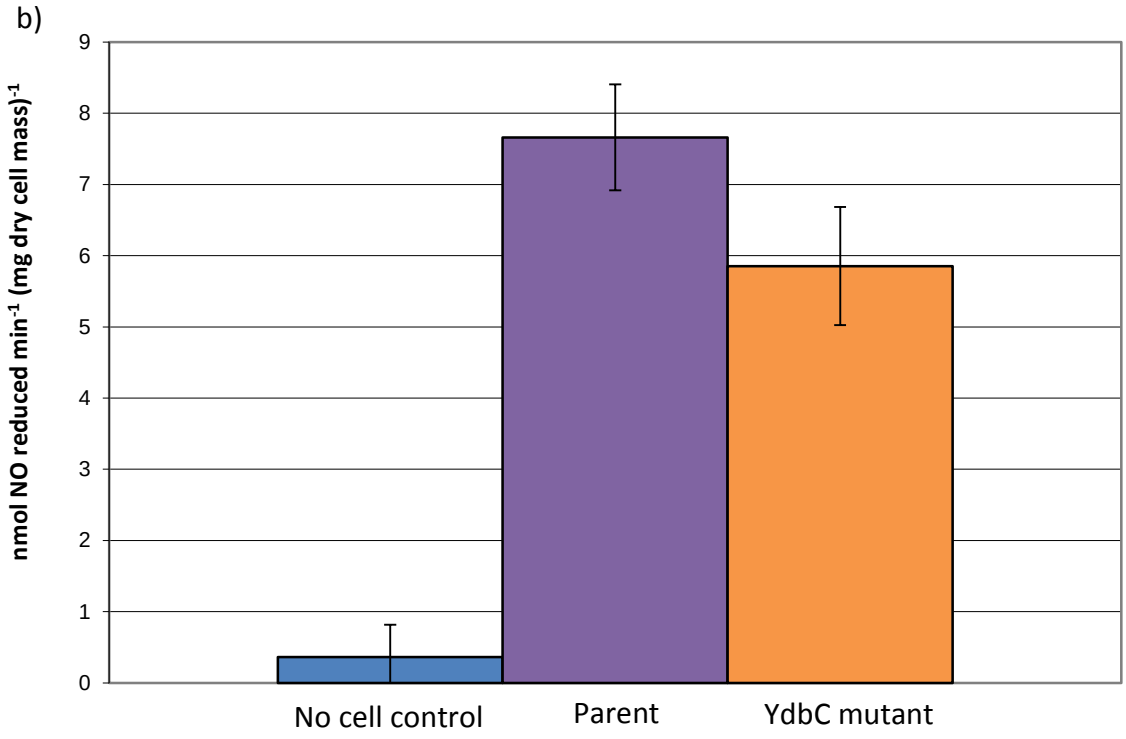
The effect of mutations on the rate of NO reduction can be used to identify whether mutated genes are involved in the reduction of NO. The parent strain RK4353, strain JCB5210 deficient in known NO reductases and a *ydbC* derivative of each were grown in the presence and absence of 20 mM nitrate, harvested and washed. The initial rate of NO reduction was recorded with 150 µM NO.

The rate of NO reduction by the parent strain, RK4353, and its *ydbC* derivative were similar (Figure 4.3a), and the strain deficient in all known NO reductases, JCB5210, had a similar rate of NO reduction compared to its *ydbC* derivative (Figure 4.3b). This suggested that a *ydbC* mutation had no effect on the rate of NO reduction either by the parent strain with all the known NO reductases present or in the mutant strain in which they were absent.

Figure 4.3 Effect of a *ydbC* mutation on the rate of NO reduction.

The parent strain RK4353 (red bar), its *ydbC* derivative (green bar), the strain deficient in all known NO reductases JCB5210 (purple bar), and its *ydbC* derivative (orange bar) were grown in the presence of 20 mM nitrate, washed, harvested and assayed for NO reduction activity with 150 μM NO. a) RK4353 and its *ydbC* derivative b) JCB5210 and its *ydbC* derivative. The x-axis shows the rate of NO reduction in nmol NO reduced per min per mg of dry cell mass in this and subsequent figures. The error bars show the standard deviation of 3 replicates.


a)
nmol NO reduced min-1 (mg dry cell mass)-1
0
1
2
3
4
5
6
7
No cell control
Parent
YdbC mutant


b)
nmol NO reduced min-1 (mg dry cell mass)-1
0
1
2
3
4
5
6
7
8
9
No cell control
Parent
YdbC mutant

Rate of NO reduction using a more sensitive electrode

To confirm that *ydbC* is not the unidentified NO reductase, an alternative NO electrode, a modified Clark-type oxygen electrode system was used that is capable of measuring much lower NO concentrations than those previously used. The parent strain for these experiments JCB5210 (deficient in all known NO reductases), and its *ydbC* derivative were grown in the presence of 5 mM nitrate, harvested, washed and assayed for NO reduction activity. PROLI NONOate was used as a source of nitric oxide at a concentration of 500 nM to provide an NO concentration of 1000 nM as one molecule of PROLI NONOate produces two molecules of NO. Again the parent strain and its *ydbC* mutant derivative showed similar rates of NO reduction (Figure 4.4). A *ydbC* mutation had no effect on the rate of NO reduction. This confirmed that YdbC is not the unidentified NO reductase.

This sensitive electrode was also used to provide an estimate for the $K_M^{app.}$ of the unidentified NO reductase using the strain deficient in all known NO reductases. It was thought that the unidentified NO reductase may have a low $K_M^{app.}$ for nitric oxide. Strain JCB5210 was grown and assayed as before. Varying concentrations of nitric oxide were used from 620 nM to 5000 nM (Figure 4.5). An estimate for $K_M^{app.}$ was calculated using SigmaPlot, using a non-linear regression, global curve fitting programme and a single rectangular hyperbolic curve. A value for $K_M^{app.}$ of 723.1 +/- 514.8 was calculated, the standard error for this estimation was large suggesting this wasn't the best model for the data, the data gave an R^2 value of 0.9376, an adjusted R^2 value of 0.8791 and a standard error of the estimate of 4.5300.

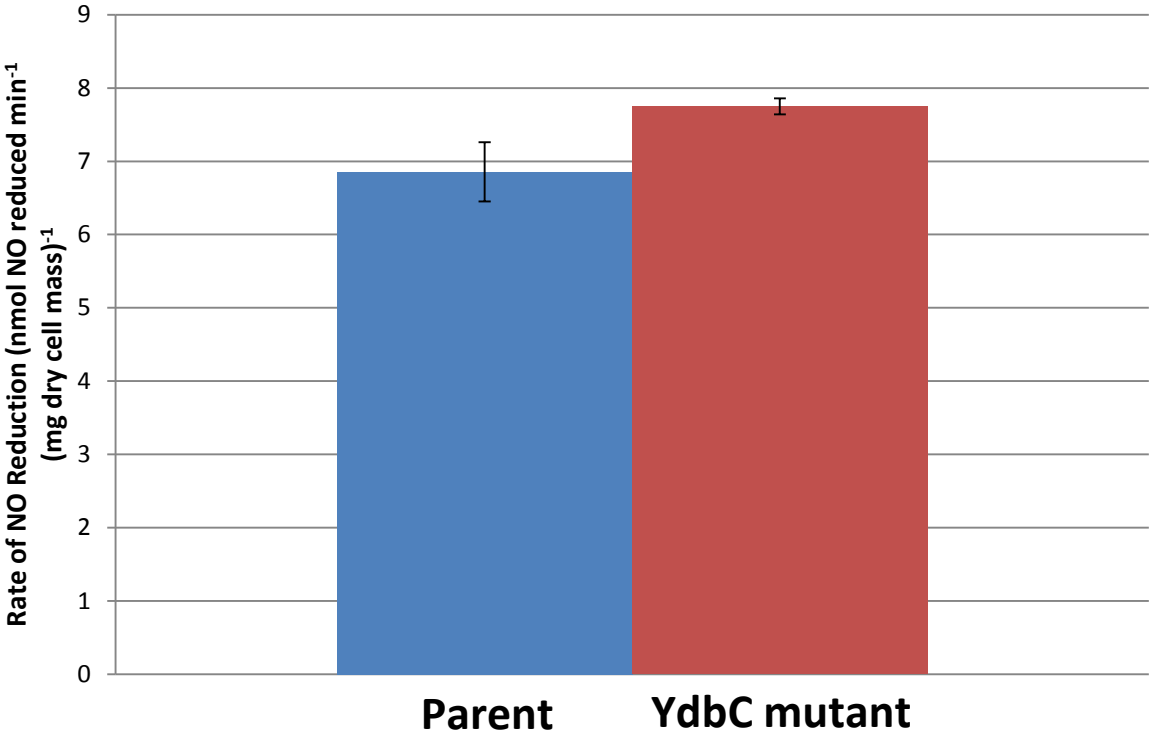


Figure 4.4 Effect of a *ydbC* mutation on the rate of NO reduction using a lower concentration of nitric oxide.

The parent strain, JCB5210 (Blue bar), and its *ydbC* derivative (Red bar) were grown anaerobically in the presence of 5 mM nitrate, harvested, washed and assayed for NO reduction activity with 1000 nM NO. The error bars in this and subsequent figures show the standard deviation of 3 replicates.

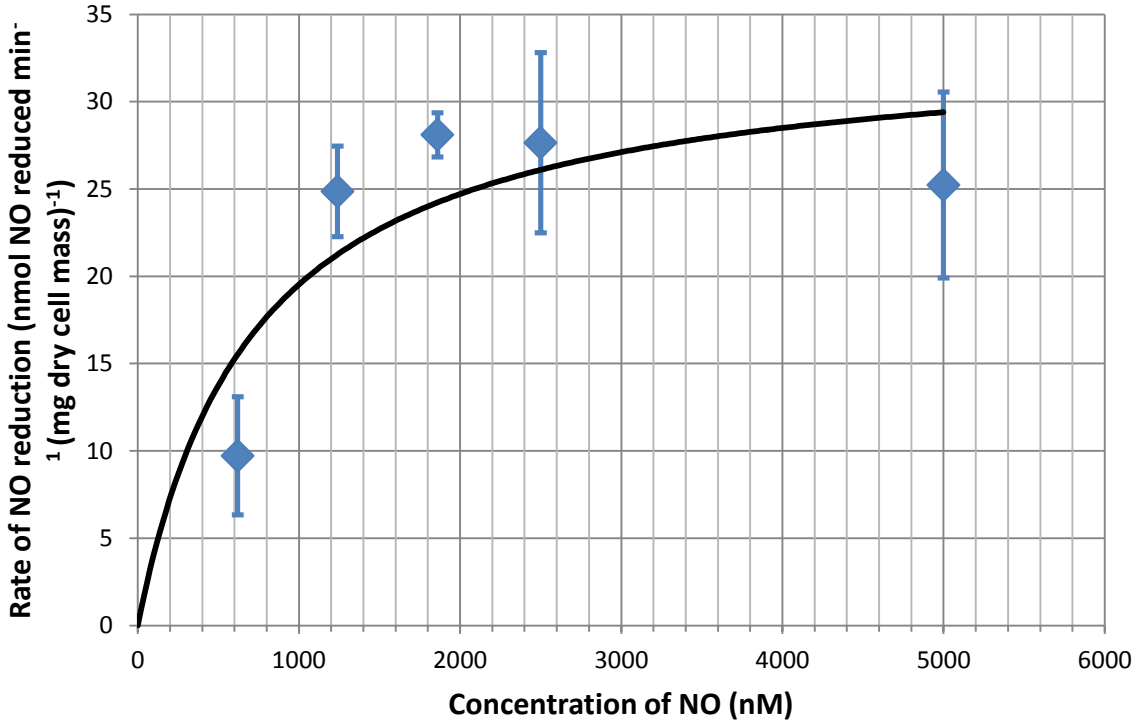


Figure 4.5 Rate of NO reduction to estimate the $K_M^{app.}$ of the uncharacterized NO reductase, NO concentration range 620 nM to 5000 nM.

The parent strain JCB5210 was grown anaerobically in the presence of 5 mM nitrate, harvested, washed and assayed for NO reduction activity using a range of concentrations for 620 nM to 5000 nM NO. The points are an average value of 3 repeats and the error bars show the standard deviation of the data. The data was analysed using the Michaelis-Menton equation. The black line shows the rectangular hyperbolic curve of the data. The value for $K_M^{app.}$ was generated using SigmaPlot.

With 5000 nM NO, the rate of NO reduction was lower than the rate detected with 2500 nM, clearly deviating from that predicted by the classic Michaelis-Menton description, showing apparent substrate inhibition. This lower rate may possibly be because high concentrations of NO caused excessive nitrosative damage, showing apparent substrate inhibition. Although it was not possible with the small amount of data shown here, more data would allow the data to be fitted to the general equation for substrate inhibition at a single site, shown below:

$$v = \frac{V_{max} \text{ x } [NO]}{K_M + [NO] \text{ x } (1 + \left(\frac{[NO]}{K_i}\right))}$$

Lower concentrations of NO (100 nM to 1000 nM) were used to determine if lower values could provide a more accurate estimate for $K_M^{app.}$.(Figure 4.6). An estimate for $K_M^{app.}$ was calculated using SigmaPlot, using a non-linear regression, global curve fitting programme and a single rectangular hyperbolic curve. A value for $K_M^{app.}$ of 709.8 +/- 79.7 was calculated. The data gave an R^2 value of 0.9975, an adjusted R^2 value of 0.9970 and a standard error of the estimate of 0.1449. The lower concentrations give a much better fit of the data to the model.

Estimation of $K_M^{app.}$ of unidentified NO reductase using non Michaelis–Menten kinetics

Although the rate of NO reduction appears to follow Michaelis-Menten kinetics at low concentrations, the decrease in the rate of reduction at higher

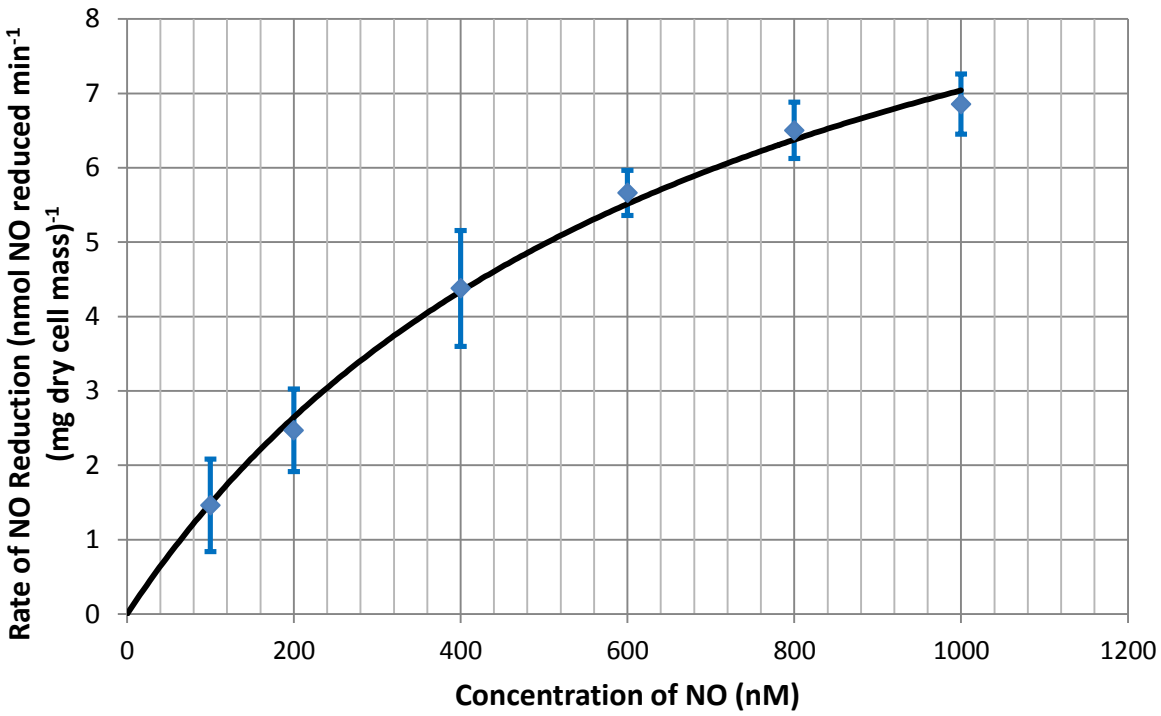


Figure 4.6 Rate of NO reduction to estimate the $K_M^{app.}$ of the uncharacterized NO reductase, NO concentration range 100 nM to 1000 nM.

The parent strain JCB5210 was grown anaerobically in the presence of 5 mM nitrate, harvested, washed and assayed for NO reduction activity using a range of concentrations for 100 nM to 1000 nM NO. The error bars show the standard deviation of 3 repeats. The data was analysed using the Michaelis-Menton equation. The black line shows the rectangular hyperbolic curve of the data. The value for $K_M^{app.}$ was generated using SigmaPlot.

concentrations suggests otherwise. In a study analysing the steady-state kinetics of the NO reductase activity in *Paracoccus denitrificans* (Girsch and de Vries, 1997), a sigmoidal relationship was shown between rate of NO reduction and NO concentration. They showed the same substrate inhibition at high NO concentrations and suggested this was due to binding of NO to the reduced enzyme. This is consistent with a model where two molecules of NO sequentially bind to the reduced enzyme. While they estimated steady-state kinetic parameters with purified enzyme, for this data intact bacterial cultures were used. Their method was used to estimate values for two dissociation constants $K_1^{app.}$, $K_2^{app.}$ and an inhibitory constant $K_i^{app.}$ using an equation fitted to the sigmoidal plot showing rate vs [NO] using 1 µM NO (Figure 4.7) and 800 nM NO (Figure 4.8). The arbitrary values from the experiment were used to calculate the rate of NO reduction at multiple points throughout a trace. These rates were then plotted against calculated values of NO concentration based on the peak when the initial NO was added. An estimate for $V_{max}^{app.}$ was taken from the graphs. Two independent traces using 1 µM NO both gave a value for $V_{max}^{app.}$ of approximately 0.0018 arbitary units and 800 nM NO gave a value of approximately 0.0015 arbitary units. These values for $V_{max}^{app.}$ were then used to fit the data to the equation below:

$$v = Vmax/(1 + K2(\frac{1}{[NO]} + \frac{K1}{[NO]^2} + \frac{[NO]}{Ki})$$

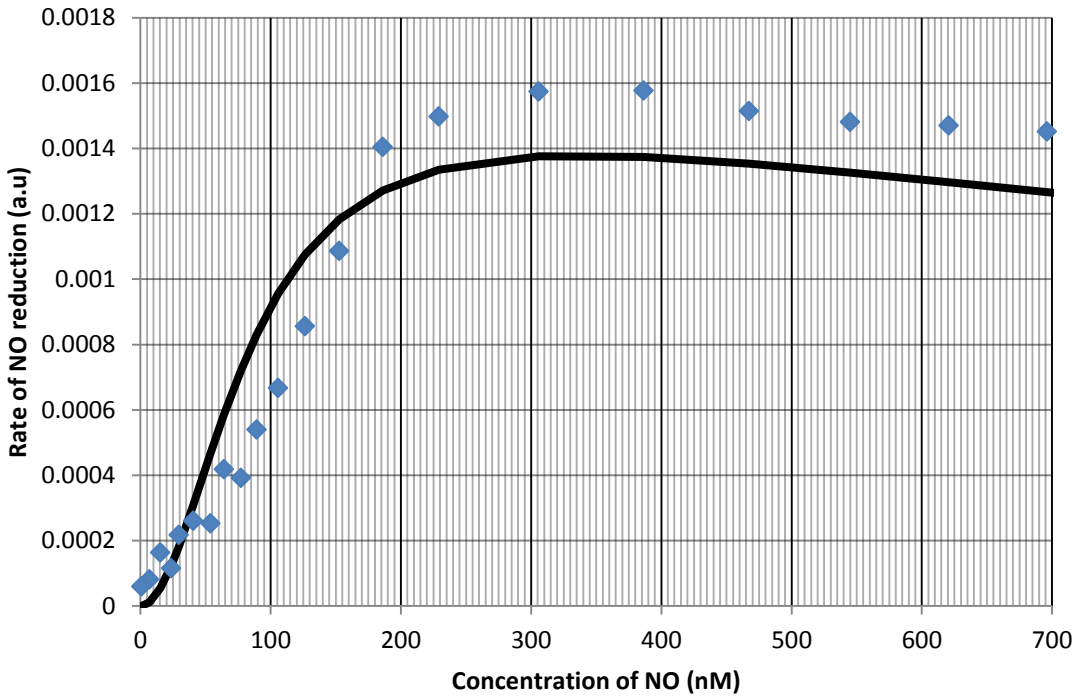


Figure 4.7 Kinetic analysis of rate of NO reduction against concentration of NO, using an initial concentration of 1 µM NO, to estimate $K_M^{app.}$ for the unidentified NO reductase.

Arbitrary values from the NO reduction assays using 1 µM NO were plotted against NO concentration calculated from the peak seen when NO was added. The points show each individual rate calculated from the original trace. The black line shows a theoretical sigmoidal curve based on the equation from Girsch and de Vries. Unfortunately, attempts to fit the data to the equation and estimate the kinetic parameters were unsuccessful.

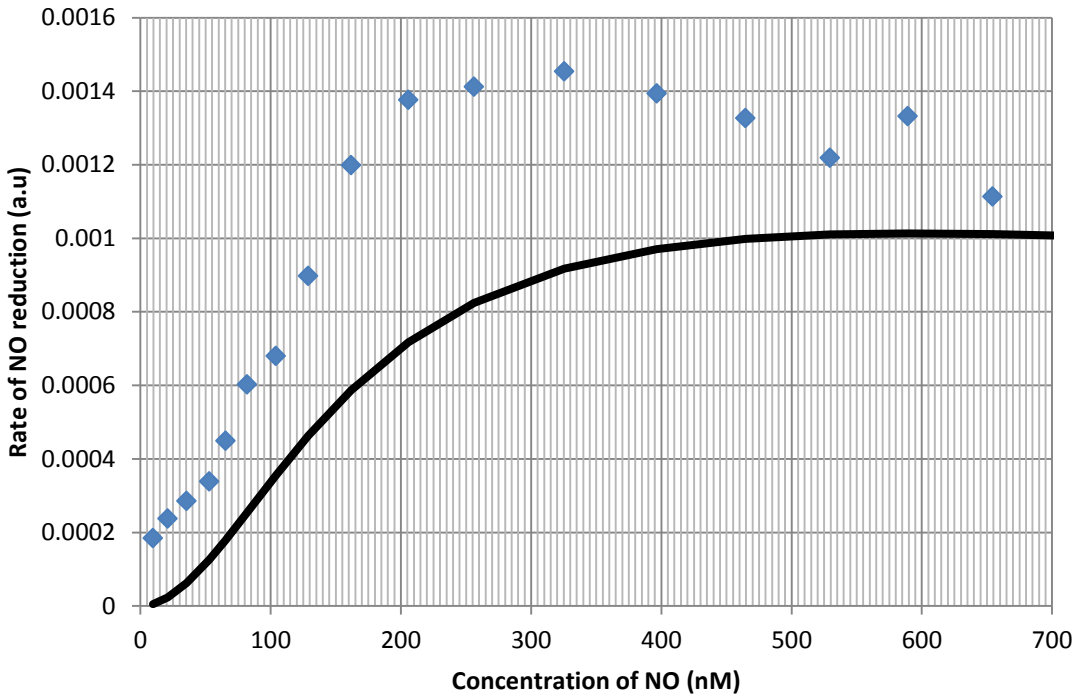


Figure 4.8 Kinetic analysis of rate of NO reduction against concentration of NO, using an initial concentration of 800 nM NO, to estimate $K_M^{app.}$ for the unidentified NO reductase.

Arbitrary values from the NO reduction assays using 800 nM NO were plotted against NO concentration calculated from the peak seen when NO was added. The points show each individual rate calculated from the original trace. The black line shows a theoretical sigmoidal curve based on the equation from Girsch and de Vries. Unfortunately, as with Figure 4.7 attempts to fit the data to the equation and estimate the kinetic parameters were unsuccessful.

Estimates for $K_1^{app.}$, $K_2^{app.}$ and $K_i^{app.}$ were attempted using Excel Solver. Using data from the trace for 1 µM NO values of 345.7 nM NO, 20.5 nM NO and 1842.4 nM NO were calculated for the three constants respectively. The data gave an R^2 value of 0.8860 indicating a poor fit. Using data from the trace for 800 nM NO values of 342.8 nM NO, 76.7 nM NO and 2152.3 nM NO were calculated for the three constants respectively. The data gave an R^2 value of 0.7862, again indicating a poor fit to the equation. The data appears to show some similarities to this sigmoidal shape but does not fit at all with the theoretical line. It is assumed in this analysis that the NO is able to equilibrate across the cell membrane, that the cytoplasmic concentration of NO is equal to that of the medium and that the NO does not react with, or isn't absorbed by any other cell component. It is possible that a repeat of the experiment, or a reanalysis of the data may yield kinetic data using this approach.

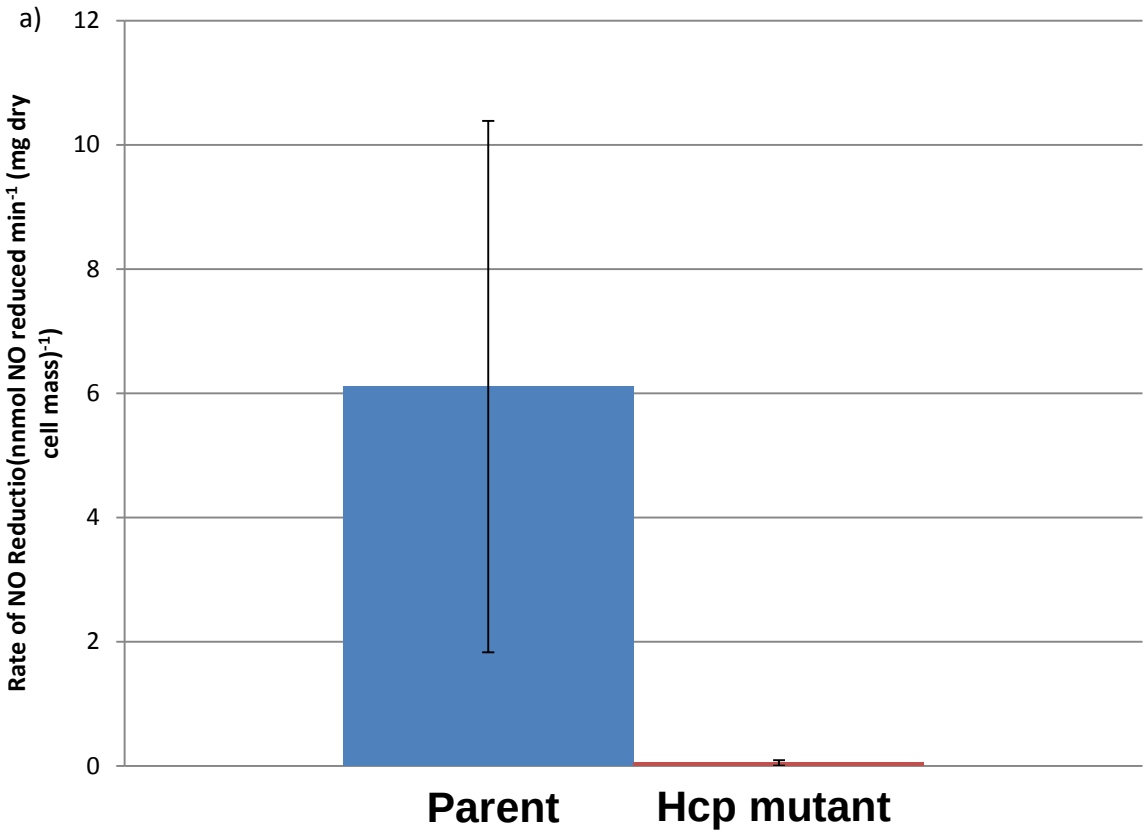
The effect of an Hcp mutation on the rate of NO reduction

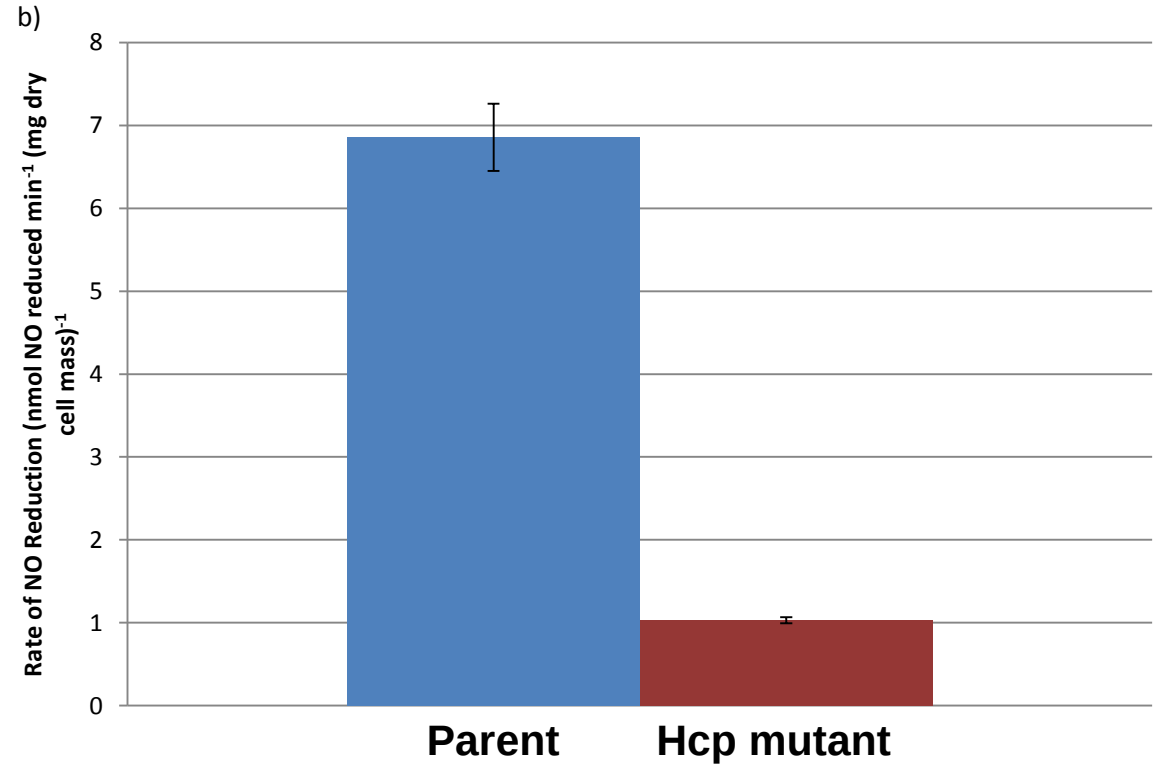
Other strains containing mutations in different genes implicated in the nitrosative stress response were also assayed to try to find the uncharacterized NO reductase. The parent strain for these experiments JCB5210, deficient in all known NO reductases and a *hcp* mutant derivative JCB 5250, were grown in the presence of 5 mM nitrate, harvested, washed and assayed for NO reduction activity as before using a concentration of 400 nM NO. The rate of NO reduction in an *hcp* mutant was very low compared to that seen for the parent strain (Figure 4.9a). Similar data were obtained using a concentration of 1000 nM NO, again showing deficient NO removal in the *hcp* mutant strain JCB 5250 (Figure 4.9b). A strain deficient in Hcp appears to be unable to remove NO at the same rate as in

an Hcp^+ strain. This suggests that Hcp plays an important role in the removal of nitric oxide.

Figure 4.9 Effect of an *hcp* mutation on the rate of NO reduction.

The parent strain JCB5210 (blue bar), and the *hcp* mutant derivative JCB5250 (red bar), were grown anaerobically in the presence of 5 mM nitrate, harvested, washed and assayed for NO reduction activity in the presence of 400 nM NO (a). This experiment was also repeated with 1000 nM NO giving similar data (b).


a)
12
10
8
6
4
2
0
Rate of NO Reductio(nnmol NO reduced min-1 (mg dry cell mass)-1)
Parent
Hcp mutant


b)
8
7
6
5
4
3
2
1
0
Rate of NO Reduction (nmol NO reduced min-1 (mg dry cell mass)-1
Parent
Hcp mutant

To confirm that an Hcp mutation results in a lower rate of NO reduction, an Hmp^+ parent strain, JCB5232, and an isogenic *hcp* mutant, JCB5242, were grown and assayed as before. The rate of NO reduction was lower in the *hcp* mutant than in the parent strain (Figure 4.10). This further confirms that Hcp plays an important role in the removal of nitric oxide even if it is not the NO reductase itself. The rate of NO reduction by the Hmp^+ Hcp^- mutant strain was slightly higher than the Hmp^+ Hcp^- strain JCB5250. This is most likely due to the slight rate of NO reduction by Hmp. This suggests that Hmp shows minimal activity under anaerobic conditions.

The effect of FNR on the rate of NO reduction

To investigate that the unknown NO reductase is important under anaerobic conditions, the parent strain, JCB5210, was grown under aerobic conditions, harvested and assayed for NO reduction activity. Under aerobic conditions the rate of NO reduction was low compared to under anaerobic conditions (Figure 4.11). To confirm that the NO reductase we are investigating is expressed anaerobically and not aerobically an FNR mutant strain, JCB5251, was grown in the presence of 5 mM nitrate and also assayed for NO reduction activity. The rate of NO reduction in the FNR mutant was low compared to the parent strain, JCB5210 (Figure 4.12). Rates of NO reduction by an FNR mutant were similar to those seen by the parent strain under aerobic conditions. This suggests that the expression of the unidentified NO reductase is under the control of FNR in anaerobic conditions.

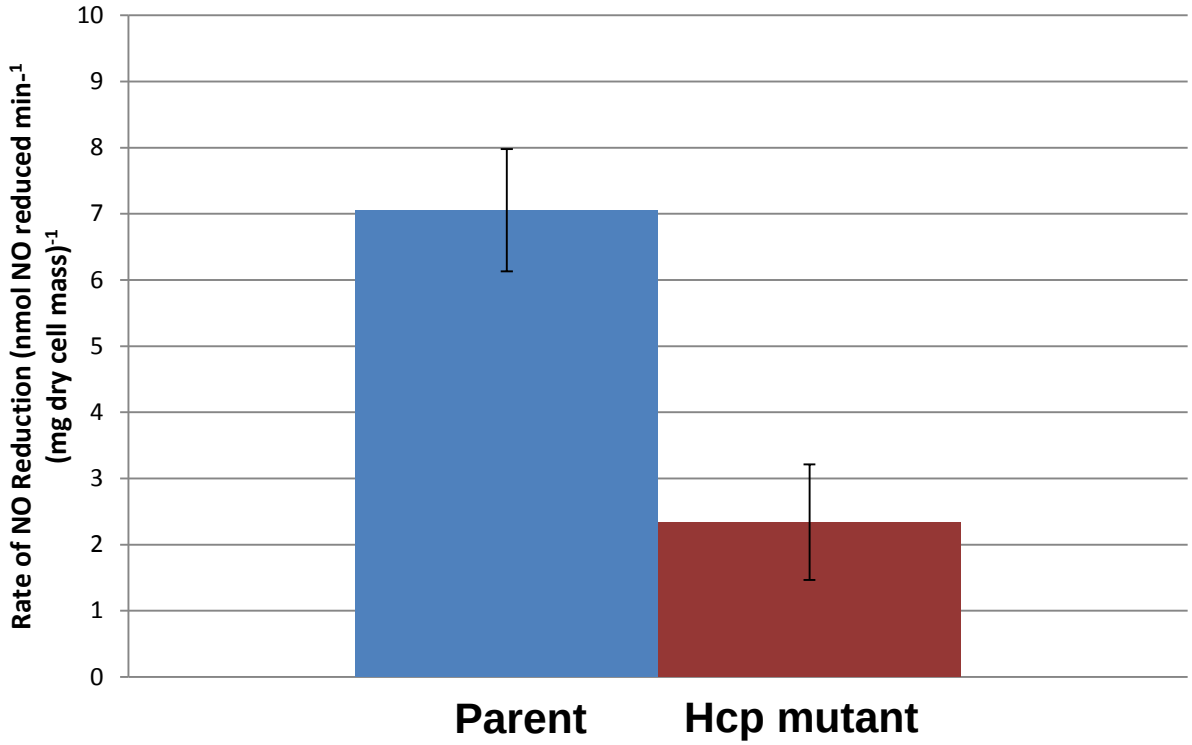


Figure 4.10 Effect of an Hcp mutation, in an Hmp^+ background, on the rate of NO reduction.

An Hmp^+ parent strain, JCB5232 (Blue bar), and the isogenic *hcp* mutant, JCB5242 (Red bar) were grown anaerobically in the presence of 5 mM nitrate and assayed for NO reduction activity.

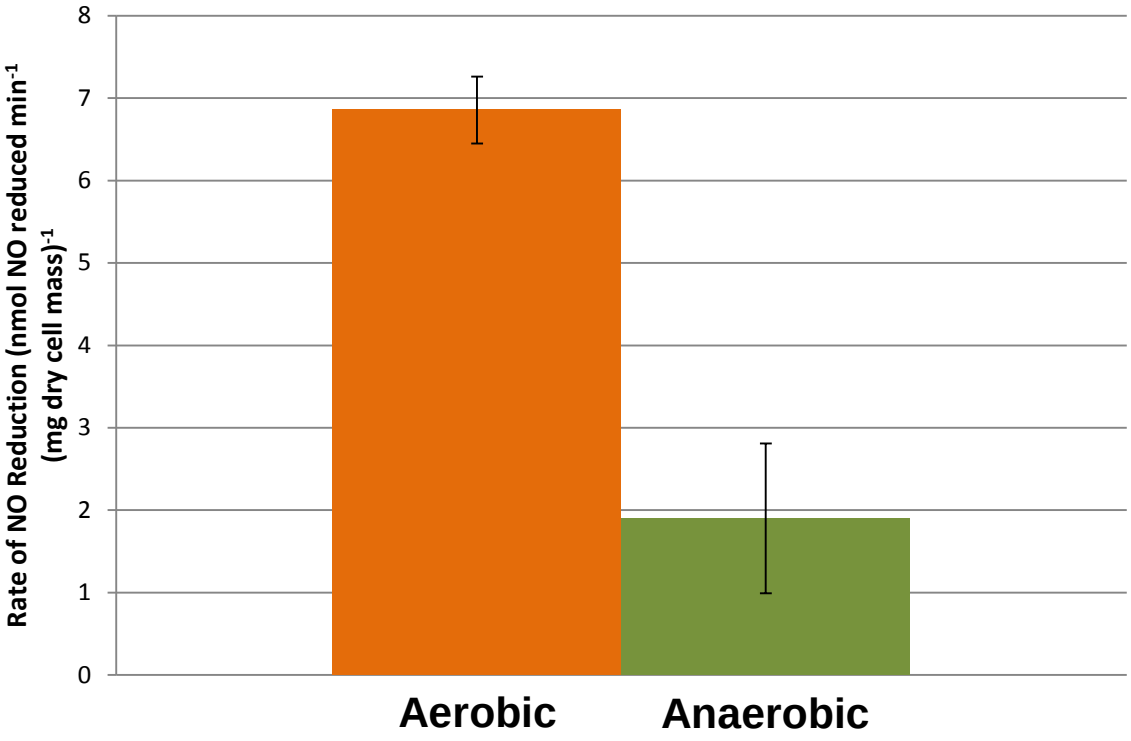


Figure 4.11 Effect of aerobic growth on the rate of NO reduction.

The parent strain JCB5210 was grown aerobically (green bar) and anaerobically (orange bar) in the presence of 5 mM nitrate.

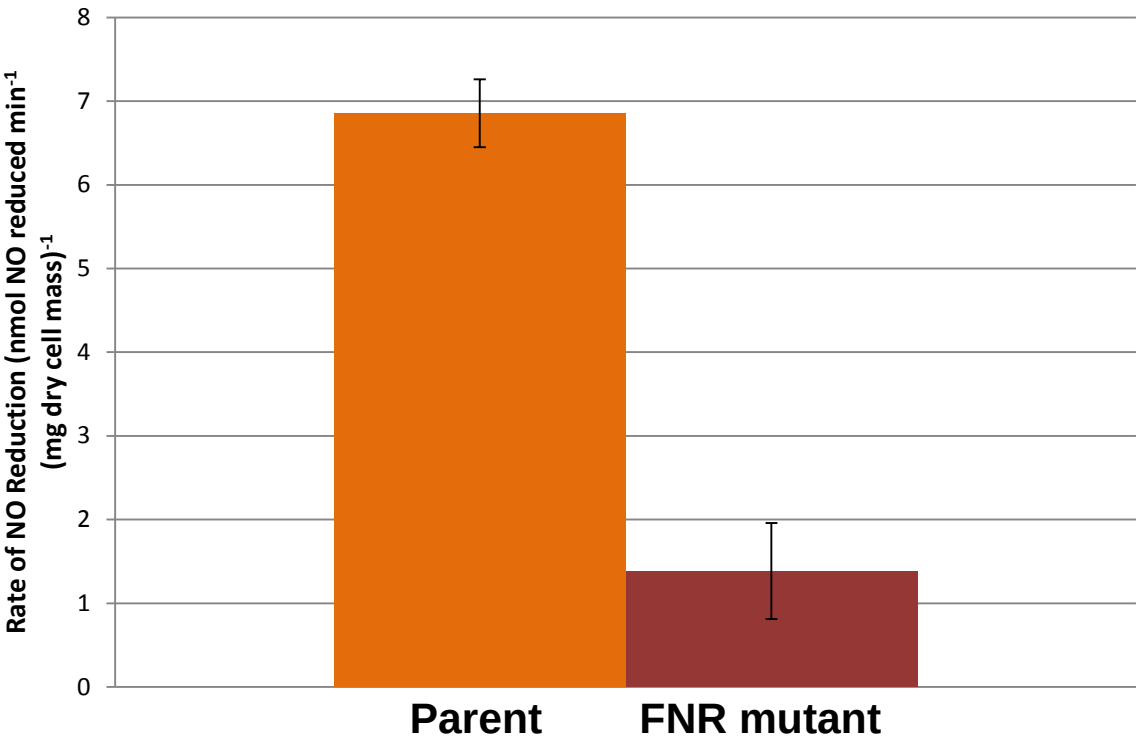


Figure 4.12 Effect of an FNR mutation on the rate of NO reduction.

The FNR mutant derivative JCB5251 (red bar) was grown anaerobically in the presence of 5 mM nitrate, harvested and assayed for NO reduction activity. Rates were compared to those of the parent strain JCB5210 (orange bar).

CHAPTER 5 – DISCUSSION

The function of Hmp under anaerobic conditions with nitrosative stress

Hmp has been reported to play a key role in the nitrosative stress response under anaerobic conditions (Corker and Poole, 2003). However, experiments discussed here suggest that Hmp is practically irrelevant under these conditions. An Hmp mutation had no effect on growth under anaerobic conditions in the presence of nitrosative stress. Hmp was unable to protect an Hcp mutant from damage due to nitrosative stress and in another experiment was only capable of very slight protection. An Hmp mutation had no effect on transcription from the *hcp* and *hmp* promoters, suggesting a lack of Hmp has no effect on the levels of NO in the cytoplasm. Also, the rate of NO reduction seen in an Hmp mutant compared to an isogenic Hmp^+ strain is only very slightly lower. If Hmp played a key role in removing nitric oxide under these conditions then the Hmp^- strain would have been expected to show a clear growth phenotype.

The function of Hcp under anaerobic conditions with nitrosative stress

Effect of loss of Hcp on growth

Unlike Hmp, an *hcp* mutation provides a clear phenotype in the absence of all known NO reductases. Although Hcp has been characterized as a Hydroxylamine reductase (Wolfe *et al*., 2002), previous work (Squire, PhD Thesis, 2009 and Vine, PhD Thesis 2011) along with the results presented in this study make this possibility unlikely. During anaerobic growth, an *hcp* mutant resulted in inhibited growth in the presence of nitrosative stress (Vine, 2011), this has also been confirmed in this project. Also, the addition of a functional Hcp-Hcr

operon from a plasmid was capable of restoring the inhibited growth seen in an Hcp mutant in the presence of nitrosative stress. This suggests that Hcp plays a key role in the nitrosative stress response, although the exact nature of this role is unclear.

Further experiments on this strain involving ferrous sulphate revealed that the inhibited growth in the presence of nitrosative stress can be relieved by the presence of ferrous ions. Although the ferrous ions may simply chelate nitric oxide stopping it from causing damage to the cell and allowing growth, it is also possible that the iron also is required for restoration of iron-sulphur clusters damaged by nitrosative stress exposure in the absence of Hcp. This was supported by the ability of ferrous ions to restore growth 2 hours following the onset of nitrosative stress, at which point any damage to the bacteria would most likely have already occurred. An Hcp mutation has also been reported to inhibit growth even in the absence of nitrosative stress (Vine, 2011). This inhibited growth has also been restored by the presence of Hcp-Hcr from a plasmid. Ferrous ions were again capable of restoring this inhibited growth, this also supports the idea that the ferrous ions are not simply mopping up NO, as under these conditions there was no NO present. This implicates Hcp in a role involving iron-sulphur clusters, either for their protection or their repair.

Even in the absence of the other mutations, an *hcp* mutant shows higher sensitivity to nitrosative stress, this time induced by a direct nitric oxide bolus. Although such a clear phenotype is not seen as when all the known NO reductases and Hcp are deleted, the effect of the lack of Hcp can still be seen. This again suggests that Hcp plays a key role in the response to nitrosative stress.

Effect of loss of Hcp on regulation by NsrR

Another clear phenotype of an *hcp* mutant strain is the high β-galactosidase activity, due to transcription from the *hcp* and *hmp* promoters, even in the absence of nitrosative stress. This high activity is due to relief of repression by NsrR. It appears, that in an Hcp mutant both in the presence and absence of nitrosative stress, NsrR is inactive and repression of the two promoters is relieved. As NsrR is an iron-sulphur cluster it may be possible that Hcp is involved in the generation or activation of functional NsrR. This is consistent with the results discussed above, that ferrous irons can restore growth inhibition seen in an *hcp* mutant, in the presence and absence of nitrosative stress. Further β-galactosidase experiments to determine if ferrous ions can restore the repression of the promoters by NsrR are required.

Effect of loss of Hcp on aconitase and fumarase activity

Other iron-sulphur cluster containing proteins are also more sensitive to nitrosative stress in an Hcp mutant. For example, the enzyme fumarase has much lower activity in an Hcp mutant in the presence of nitrosative stress and the enzyme aconitase has much lower activity in an Hcp mutant both in the presence and absence of nitrosative stress. Both results suggest that Hcp plays a role in the protection or repair of iron-sulphur clusters. In future work, further aconitase and fumarase assays to see if ferrous ions are capable of restoring activity could be performed, as well as complementation of activity with the Hcp-Hcr plasmid.

Effect of loss of Hcp on the rate of NO reduction

Another clear phenotype of an Hcp mutant strain is observed when measuring NO reductase activity. A strain deficient in all known NO reductases still shows a similar rate of NO reduction to the parent strain, suggesting that another key system of NO removal remains to be characterized. The introduction of an Hcp mutation to this strain results in a clear loss of NO reduction. This suggests that Hcp may itself be the unidentified NO reductase. However, this combined with the previous data makes it possible that Hcp regulates or repairs the uncharacterized NO reductase, or possibly Hcp regulates an iron-sulphur cluster protein that regulates the NO reductase. What is clear is that an Hcp mutation severely damages the ability of *E. coli* to resist nitrosative stress. An Hcp mutant is unable to grow in the presence of nitrosative stress and growth is also inhibited in its absence. Regulation of other genes important in the nitrosative stress response are affected and key enzymes required by the cell are more easily damaged.

The function of YtfE under anaerobic conditions with nitrosative stress

A mutation of YtfE is capable of restoring the inhibited growth seen in an *hcp* mutant. It was suggested this was due to YtfE producing a toxic product that is particularly damaging in an Hcp deficient strain. Suggestions for this toxic product included ferrous ions. This project ruled out this Fe^{2+} as the toxic product and instead identified the toxic product as nitric oxide itself, by the fact that PTIO, an NO scavenger was capable of relieving the inhibition in the $YtfE^{+}$ strain. YtfE is known to repair iron-sulphur clusters damaged by nitrosative stress (Justino *et al.*, 2005; Justino *et al*., 2006; Justino *et al.*, 2007; Overton *et al.*, 2008; Vine *et*

al., 2010), however, the mechanism of repair is unknown. The generation of NO by YtfE suggests that it may remove the NO from the nitrosylated iron-cluster or remove the nitrosylated iron-cluster and release the nitric oxide without releasing the Fe^{2+}. If YtfE does remove the damaged iron centre, Hcp may be involved in recycling the Fe^{2+} to restore the cluster.

As well as a mutation of *ytfE* partially restoring growth of an *hcp* mutant, a ytfE mutation can also partially restore regulation by NsrR in an *hcp* mutant. Again this may be due to the lower levels of nitric oxide in a *ytfE* strain. However, this does not explain the partial restoration of repression in the absence of nitrosative stress. A ytfE mutation has no effect on regulation by NsrR in a Hcp^{+} background. The effect of deleting YtfE is only seen in strains where Hcp is absent.

Kinetics of the rate of NO reduction

The graph of concentration of substrate against rate for each strain shows that the rate of NO reduction is dependent on the concentration of NO added. Both the parent strain, RK4353, and the strain deficient in all known NO reductases give the same curve so the V_{max} and $K_M^{app.}$ for both strains appears to be similar. It is important to determine the kinetics of the rate of NO reduction in order to determine if the reaction is chemical or enzymic. As the data appeared to follow Michaelis–Menten kinetics rather than a linear relationship it can be concluded that the NO is being reduced by an enzyme and is not being chemically removed. This means at least one other NO reductase remains to be characterised.

YdbC ruled out as the unidentified NO reductase

The rates of NO reduction seen for the parent strain, RK4353, the strain deficient in all known NO reductases, JCB5210, and their *ydbC* derivatives all showed a similar rate of NO reduction on two different electrodes and with two very different concentrations of nitric oxide. This rules out YdbC as an NO reductase as a lower rate of NO reduction should have been observed in both the *ydbC* mutants.

$K_M^{app.}$ of the unidentified NO reductase

A more sensitive electrode was used to provide an estimate for the value of $K_M^{app.}$ for this unidentified NO reductase. Assays using higher concentrations of NO gave more variable data, whereas the lower concentrations gave more reproducible data. Both sets of data gave similar estimates for the value of $K_M^{app.}$ with the higher concentrations of NO giving a value of 723.1 nM NO and the lower concentrations of NO giving an value of 709.8 nM NO. This suggests that the uncharacterized reductase has a high affinity for NO. Both estimates were generated by fitting the data to the standard Michaelis-Menten equation, as the R^2 value of the higher concentrations was lower than for the lower concentrations it suggest that at higher concentrations of NO Michaelis-Menten kinetics is not followed. It is possible that the reductase shows substrate inhibition in the presence of high concentrations of NO but more data is required to fit to the equation for substrate inhibition.

An alternative kinetic analysis was used (Girsch and de Vries, 1997) that has previously been shown to be consistent with a model of sequential binding of two molecules of NO to the reduced NO reductase from *Paracoccus denitrificans*.

Our data follows a similar sigmoidal relationship between rate of NO reduction and NO concentration and the same apparent substrate inhibition at high concentrations. Attempts to fit the data to the equation of Girsch and de Vries gave values for the parameters of two dissociation constants $K_1^{app.}$, $K_2^{app.}$ and an inhibitory constant $K_i^{app.}$ of 345.7 nM NO, 20.5 nM NO and 1842.4 nM NO respectively. These similarities may suggest the uncharacterised NO reductase uses a similar model, sequential binding two molecules of NO. However, it should be stressed that the data fitting parameter R2 indicate a poor fit of the data to the theoretical curves. Therefore, interpretation of the data is not valid is not valid at this stage without a repeat of the experiment or re-analysis of the data.

The effect of FNR on the rate of NO reduction

Under aerobic conditions the rate of NO reduction was low compared to under anaerobic condition and the rate of NO reduction in the FNR mutant was low compared to the parent strain. An FNR mutant showed rates of NO reduction similar to those seen by the parent strain under aerobic conditions. This suggests that the unidentified NO reductase is expressed anaerobically and is regulated by FNR.

Differences between nitrate, nitrite and nitric oxide as a source of nitrosative stress

In the parent strains, not sensitive to nitrosative stress, relief or repression of NsrR occurred within 2 following the addition of nitrite as it is rapidly converted to nitric oxide. NsrR binds NO and relieves repression of transcription. This was not seen within 2 hours with nitrate as nitrate inhibits the reduction of nitrite to nitric oxide, until all the nitrate has been reduced, no NO will be produced to relieve

repression of transcription. Relief of repression was not seen 2 hours following the addition of NO either, this may be due to the inability of NO to be effectively transported across the membrane.

Future Experiments

YtfE, like Hcp, will also be expressed from a plasmid to see if the phenotype can be complemented. Recombinant His-tagged forms of fumarase and aconitase are also being prepared for overexpression and purification in the laboratory. This will allow the protein to be isolated and used for Mass Spectrometry to test whether the protein binds NO and how this state may relate to cellular growth conditions, e.g. nitrosative stress. In combination with these experiments the Hcp protein will be overexpressed from a plasmid and isolated again for use in Mass Spectrometry. The same can also be done for YtfE. Identifying if any difference between these proteins occurs that depends on the conditions they are expressed in will provide insight into the roles these proteins play under these conditions.

Further experiments are also required to determine the exact role of Hcp. Aconitase and fumarase assays to determine how the activity can be restored are key. The addition of a functional Hcp-Hcr gene from a plasmid, purified Hcp protein and ferrous ions will be investigated to see if any are capable of restoring activity of the enzymes.

Further NO reduction assays need to be completed. Strains deficient in YtfE in both an Hcp^{+} and *hcp* mutant background will provide information about how YtfE functions. NsrR mutants of the strains deficient in all known NO reductases and Hcp need to be made and then assayed to investigate if the low rate of NO

reduction seen in a *hcp* mutant is due to the inactivation of NsrR. This would suggest that Hcp is responsible for regulating NsrR, which in turn regulates the uncharacterized NO reductase.

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