The regulation of genes of unknown function implicated in nitrosative stress tolerance in *Escherichia coli* K-12

by

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Synopsis

This study was designed to determine the regulatory network that controls expression from two *Escherichia coli* K-12 promoters, p*yeaR* and p*ogt*, during anaerobic growth. These promoters were identified from transcriptomic studies as being positively regulated by NarL independently of FNR, the master regulator of anaerobic respiration. Biochemical and genetic analyses presented in this study confirmed that expression from both the *yeaR* and *ogt* promoters is dependent upon NarL, which binds to a single site in the *yeaR* promoter and two sites in the *ogt* promoter. The nucleoid-associated protein, Fis, repressed transcription from both promoters, especially in rich medium, by binding to sites that overlap the NarL site, excluding the essential activator.

Both promoters were more active in the absence of functional FNR. However, mutational analysis revealed that FNR does not bind to the *yeaR* promoter region, so this effect is indirect. How the absence of functional FNR might affect NarL-dependent nitrite signalling was investigated.

The Ogt protein is known function as an O^6-alkyguanine methyltransferase. However, the functions of the gene products of *yeaR-yoaG* and another operon implicated in nitrosative stress management, *hcp-hcr,* were unknown. Strains carrying a chromosomal *yeaR-yoaG* deletion were not more sensitive to nitric oxide or hydroxylamine compared with the parental strain, suggesting that the products of this operon are not essential for dealing with these toxic nitrogen species. Conversely, a strain deleted in *hcp-hcr* was shown to be slightly more sensitive to both nitric oxide and hydroxylamine, implicating Hcp and Hcr in nitrosative stress management.

Dedicated with love and thanks to my family and to Beth

"If you are going through Hell…keep going!"
Winston Churchill

Acknowledgments

First and foremost I must thank my supervisor, Jeff Cole, who, despite me having very little laboratory experience, gave me the opportunity to complete a PhD in his research group. Jeff has been a constant driving force behind me and has provided an astonishing amount of knowledge for the past four years. My thanks must also go to Professor Steve Busby, who has helped to shape and direct my research through the duration of my project. Special thanks go to Dr Doug Browning, Dr Dave Lee and Dr Kerry Hollands who were always ready and willing to teach me *in vitro* techniques and share their considerable expertise in this area. I would like to thank all members of the Cole and Busby research groups who have known me for the last four years and have kept me going with smiles, support and endless cups of coffee. I would like to than my good friends and 'surrogate parents', Duncan and Nikki-Ann, who gave me a happy and comfortable home for the difficult period between student digs and own home. I thank my parents for their support and my brother, Andrew, and his partner, Faye, for their near constant use of the phrase "it will be worth it in the end". Finally I thank my fiancée, Beth, who, despite knowing nothing about the subject, has sat through countless practice talks, read reports and feigned interest in everything I do!

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A/C/G/T/U	adenosine/cytosine/guanine/thymidine/uracil
APS	ammonium persulphate
bp	base pairs
BSA	bovine serum albumin
ChIP	chromatin immunoprecipitation
CRP	cAMP receptor protein
CTD	carboxy-terminal domain
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxyribonucleoside 5'triphosphate (N=A,C,G or T)
ddNTP	2'3'-deoxyribonucleoside 5'triphosphate (N=A,C,G or T)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
GSNO	S-nitrosoglutathione
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl-β-Dthiogalactopyranoside
kb	kilobase
kDa	kilodalton
LB	Lennox broth
mRNA	messenger ribonucleic acid
MS	minimal salts medium
NO	nitric oxide
NOSW	nitric oxide saturated water
nt	nucleotide
NTP	nucleoside 5'-triphosphate (N=A,C,G or U)
NTD	amino-terminal domain
OD	optical density
ONPG	2-nitrophenyl-β-Dgalactopyranoside
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RNAP	RNA polymerase
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute
SAM	S-adenosylmethionine
SDS	sodium doecyl sulphate
TBS	Tris buffered saline

TE	Tris EDTA buffer
TEMED	N,N,N',N'-tetramethyethylenediamine
TMAO	trimethylamine N-oxide
Tris	tris(hydroxymethyl)aminomethane
UV	ultra violet
v/v	volume per volume
WT	wild type
w/v	weight per volume

Chapter 1

Introduction

The facultatively anaerobic bacterium *Escherichia coli*, and its life in the mammalian gut

The Gram-negative enterobacterium, *Escherichia coli*, is found in many diverse environments including the mammalian gut (Drasar *et al.*, 1966). As a result of its lifestyle, *E. coli* must be able to adapt from the aerobic environment outside the host to the anaerobic environment in the gastro-intestinal tract (Cole, 1996). Within the host, *E. coli* uses an array of alternative electron acceptors in order to respire efficiently in the absence of oxygen (Richardson, 2000). The two most powerful oxidants available in the mammalian gut are nitrate (NO_3^-) and nitrite (NO_2^-), which *E. coli* can reduce readily to ammonia (reviewed by Cole, 1996; Saul *et al.*, 1981). When inhabiting the gastrointestinal tract, *E. coli* must be able to protect itself from host defences, which generate an array of cytotoxic nitrogen-based chemicals to destroy the bacterium, as well as the toxic intermediates *E. coli* and other bacteria sharing the gastro-intestinal tract produce during anaerobic nitrate respiration (Bogdan *et al.*, 2000; Nathan, 1997; Nathan and Shiloh, 2000). Several systems have been identified in *E. coli* that reduce or detoxify nitric oxide. However, many other genes, some of unknown function, have been implicated as being involved in nitrosative stress management (Constantinidou *et al.*, 2006). This study aims to discover how *E. coli* regulates the expression of genes of unknown function that are implicated in nitrosative stress tolerance. Therefore, this chapter will describe: how and when *E. coli* is likely to encounter nitric oxide while growing in an anaerobic environment; how the bacterium is able to detect nitric oxide and its precursor molecules; the mechanisms by which *E. coli* can detoxify NO or repair the damage it has caused and how *E. coli* regulates the expression of specific genes at the transcription level.

The environmental nitrogen cycle

The element nitrogen is an essential component of many biological molecules, most importantly in proteins and nucleic acids. It is present in the environment in redox states from +5 to -3 and is inter-converted between these states by highly specialised enzymes, many of which contain metal iron cofactors (reviewed by Richardson and Watmough, 1999). The inter-conversion of nitrogen between varying oxidation states, facilitated by biological, geological and chemical catalysts, forms the basis of the global nitrogen cycle. Within this complex cycle, bacteria play a key role. Assimilation of di-nitrogen gas (N_2) into organic matter, otherwise known as nitrogen fixation, is facilitated by a more limited number of bacterial species, which possess nitrogenase enzymes. Denitrification, or the reduction of nitrate and nitrite to nitric oxide (NO), nitrous oxide (N_2O) and di-nitrogen (N_2), is a conserved respiratory pathway utilised by denitrifying bacteria (reviewed by Berks *et al.*, 1995a; Ferguson, 1998).

The reduction of nitrate to nitrite and the successive reduction of nitrite to other nitrogen oxides play a key role in the loss of fixed nitrogen from the environment. The eventual production of nitrous oxide, a greenhouse gas, from the reduction of nitric oxide by bacteria has led to world wide interest in the biogeochemical nitrogen cycle and the introduction of control on the use of *N*-fertilisers in agriculture (Butler, 2003; Lane, 2007).

The enteric bacterium *E. coli* is able to use a number of enzymes to capitalise on the presence of nitrate and other alternative electron acceptors. Using these alternatives, either in the host or in the environment, *E. coli* is able to respire efficiently in the absence of oxygen (Richardson, 2000). The ability of *E. coli* to switch from aerobic to anaerobic growth is fundamental to its life as an intestinal pathogen as well as an environmental bacterium, and is therefore the subject of intense study (Cole, 1996; Lundberg *et al.*, 2004). Although the regulation and function of many aspects of nitrate metabolism are well understood, recent insights into the endogenous generation of nitric oxide and other toxic nitrogen species during nitrite reduction has raised interest in the subject. One key aspect, which is the focus of this

study, is how *E. coli* is able to detoxify or remove the cytotoxic reactive nitrogen species such as nitric oxide that it generates itself during anaerobic nitrite metabolism (Corker and Poole, 2003). The resistance of pathogenic *E. coli* to reactive nitrogen species may also play a part in the resistance of the bacterium to host phagocytic cells that generate NO to kill engulfed bacteria (Bogdan *et al.*, 2000; Fang, 2004; Nathan and Shiloh, 2000).

Anaerobic nitrate respiration in *E. coli*

In order to respire efficiently, the bacterium must find a suitable alternative electron acceptor in order to generate energy in the form of proton motive force (reviewed by Richardson, 2000). These alternatives include nitrate, nitrite, TMAO, DMSO and fumarate. The most powerful oxidant, when oxygen in unavailable, is nitrate, which can be reduced via specific enzymes known as the nitrate reductases (Bonnefoy and Demoss, 1994; Ferguson, 1998; Richardson, 2000). The chromosome of *E. coli* encodes three distinct nitrate reductases that all catalyse the reduction of nitrate to nitrite but are synthesised under different conditions (Blattner *et al.*, 1997). This study is concerned mainly with dissimilatory nitrate reductases, which generate energy via the coupling of nitrate reduction to the generation of Δp, or proton motive force, and generate reactive nitrogen species as a consequence. *E. coli* has three dissimilatory nitrate reductases: two homologous membrane bound reductases, NAR, and one soluble periplasmic reductase, NAP (Philippot and Hojberg, 1999)(fig. 1.1).

The nitrate reductase, NarGHI

The membrane bound nitrate reductase A (NRA), also known as NarGHI, is composed of three major protein subunits: α, β and γ (encoded by *narG, H* and *I,* respectively) as well the auxiliary subunit, NarJ. The γ-subunit, encoded by *narI,* is a transmembrane protein that inserts into the cytoplasmic membrane of the cell and anchors the rest of the nitrate reductase to the membrane (Berks *et al.*, 1995b). The γ-protein is able to accept electrons donated by reduced NADH, via the quinol pool, using a high potential *b*-type haem group co-ordinated in the γ

Figure 1.1

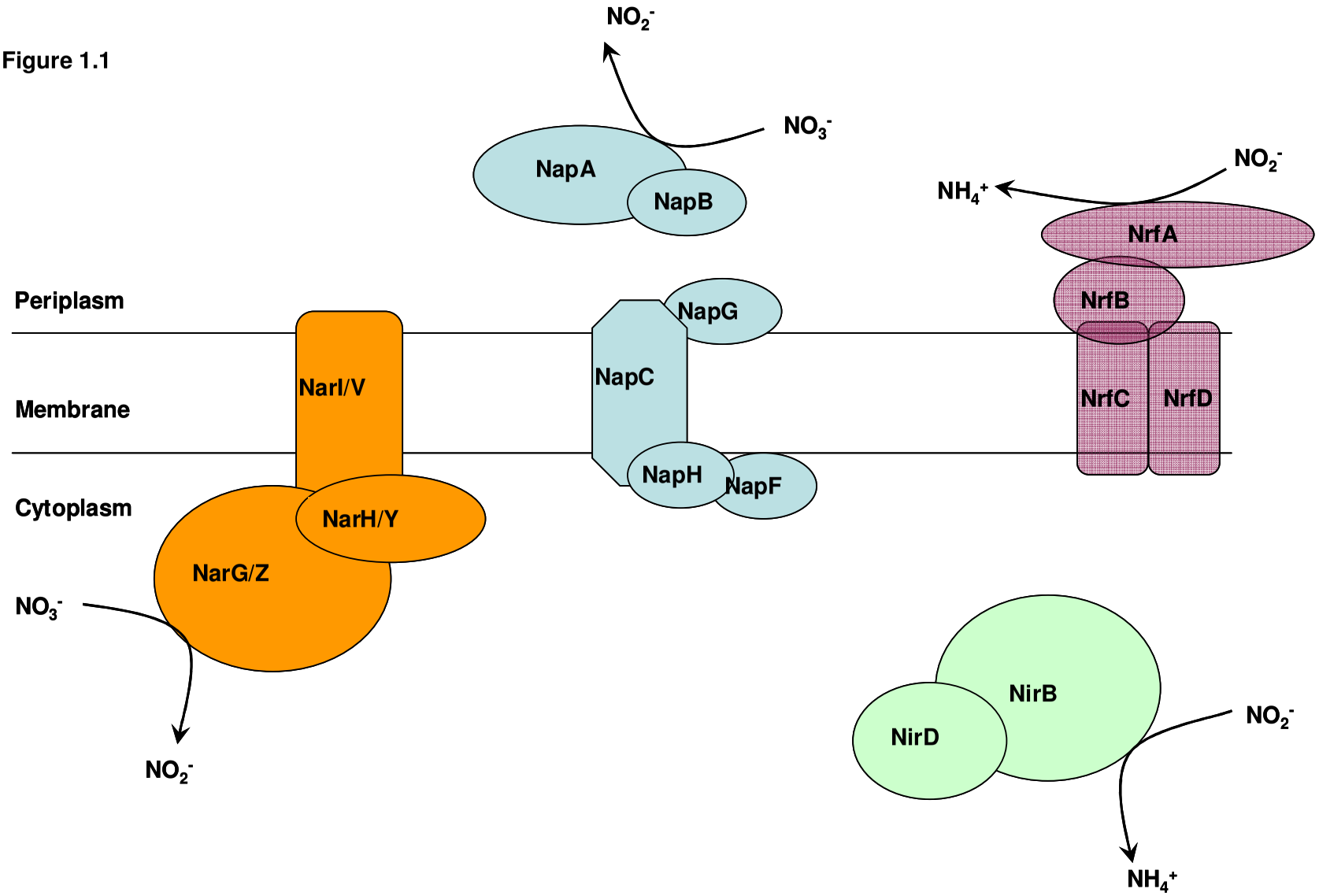
NO_2^-
NO_3^-
NapA
NapB
NO_2^-
NH_4^+
NrfA
NrfB
Periplasm
NapG
NapC
NrfC
NrfD
Membrane
NarI/V
NapH
NapF
Cytoplasm
NarH/Y
NarG/Z
NO_3^-
NO_2^-
NirB
NirD
NO_2^-
NH_4^+

Figure 1.1 Anaerobic nitrate ammonification in the cytoplasm and periplasm of *E. coli*

Two pathways for nitrate ammonification via nitrite are present in *E. coli.* The first, cytoplasmic pathway includes the nitrate reductase A encoded by *narGHI*, which reduces nitrate to nitrite in the cytoplasm, and the soluble, cytoplasmic, NADH-dependent nitrite reductase, NirB. Nitrate reduction by this pathway is coupled to energy generation by the expulsion of protons by NarGHI, however, NirB-dependent nitrite reduction is not coupled to proton translocation. The second pathway reduces nitrate to ammonia in the periplasm via the nitrate reductase, Nap, and the *c*-type cytochrome nitrite reductase, NrfA. A third nitrate reductase homologous to NarGHI, NarZYW, is also able to reduce nitrate to nitrite in the cytoplasm.

subunit (Hackett and Bragg, 1983). The reduction of the haem group and the subsequent oxidation of the quinol pool leads to the expulsion of two protons (H^+) into the periplasmic space, resulting in the generation of Δp (Garland *et al.*, 1975). This gradient of protons across the cytoplasmic membrane drives the production of ATP via ATPase (Garland *et al.*, 1975). Electrons are then passed to a lower potential haem group in the γ subunit before eventually being passed, via two iron-sulphur centres in the β subunit, to the catalytic subunit, NarG. The large globular β-subunit contains 4 iron-sulphur centres, 1 [3Fe-4S] centre and 3 [4Fe-4S] centres. The two electrons, donated by the quinol pool and passed from the γ-subunit through the β-subunit, are donated to an iron-sulphur cluster present in the α-subunit, NarG. The electrons are then donated to a covalently bound molybdopterin cofactor that catalyses the reduction of nitrate to nitrite according to the following equation: $2H^+ + NO_3^- + 2e^- \rightarrow NO_2^- + H_2O$. This coupling of nitrate reduction with the generation of Δp allows *E. coli* to respire efficiently (fig 1.2).

The nitrate reductase, NarZWY

The second membrane-bound nitrate reductase, nitrate reductase Z (NRZ), is a homologue of the NarGHI and is thus termed NarZYW (Iobbi *et al.*, 1987). This nitrate reductase shows considerable similarity to NarGHI and has been shown to have a similar αβγ-subunit arrangement (Blasco *et al.*, 1990). NarZYW has also been shown to contain a molybdopterin cofactor and an iron-sulphur centre in the α- subunit, similar to NarG (Blasco *et al.*, 1990). The action of the two enzymes has been shown to be functionally similar by creating a protein 'chimera' by associating the β and γ subunits of NarZYW with the α-subunit of NarGHI to give an active and functional membrane bound nitrate reductase (Blasco *et al.*, 1992). Unlike the expression of the major nitrate reductase-encoding genes, *narGHI,* the expression of *narZYW* is insensitive to the presence or absence of oxygen or nitrate and is synthesised at a low level during exponential growth (Iobbi *et al.*, 1987). However, this

Figure 1.2

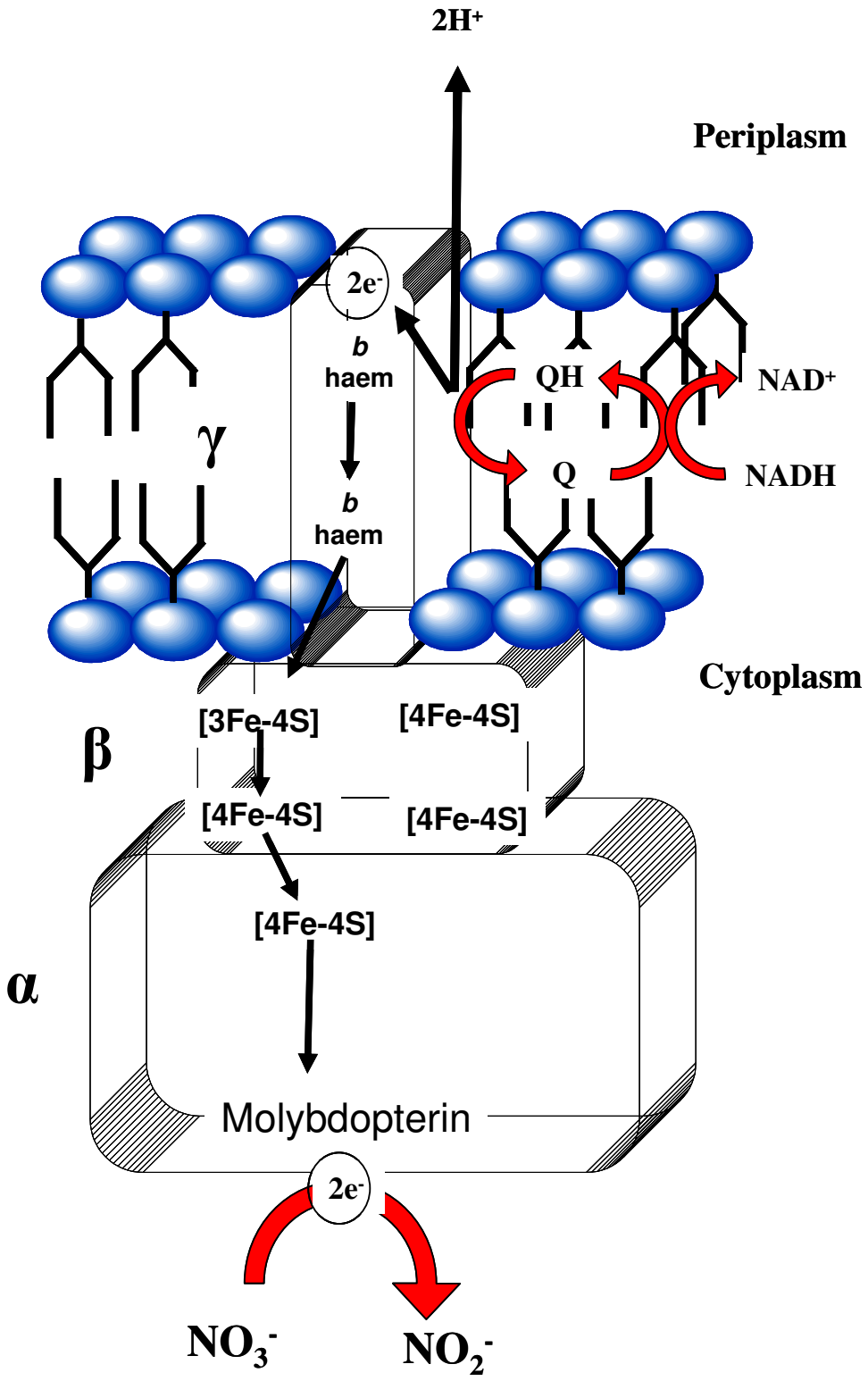
2H+
Periplasm
2e-
b
haem
QH
NAD+
γ
Q
NADH
b
haem
Cytoplasm
[3Fe-4S]
[4Fe-4S]
β
[4Fe-4S]
[4Fe-4S]
[4Fe-4S]
α
Molybdopterin
2e-
NO3-
NO2-

Figure 1.2. Electron transfer and nitrate reduction by the nitrate reductase, NarGHI

Electrons donated from NADH via the quinol pool are passed to the γ-subunit of the nitrate reductase A. These electrons are then transported via the two b-type haems contained in the γ-subunit, through the iron-sulphur centres of the β-subunit to the molybdopterin cofactor of the catalytic α-subunit, NarG. Nitrate reduction to nitrite occurs in the cytoplasm and is coupled to the expulsion of $2H^+$ into the periplasm and the generation of Δp.

expression is increased upon entry into stationary phase and is highly, but not entirely dependent upon the alternative sigma factor, RpoS, suggesting that this nitrate reductase might be synthesised to allow the bacterium to utilise nitrate under stress-associated conditions (Chang *et al.*, 1999).

The periplasmic nitrate reductase, Nap

The third, soluble, nitrate reductase of *E. coli* is the periplasmic nitrate reductase, Nap. Although the chemical reduction of nitrate to nitrite is conserved, the structure of the protein and the mechanism of the reduction are considerably different. This protein is widespread among bacteria that inhabit vastly different environments in soil and sediment (Carter *et al.*, 1995), as well as in a number of denitrifiers, non-sulphur photosynthetic bacteria and enterobacteria (Potter *et al.*, 2001). The periplasmic nitrate reductase is a molybdenum-containing enzyme complex that is active in the periplasm of *E. coli* (Potter and Cole, 1999). All of the *nap* genes are encoded by the operon *napFDAGHBC* and are expressed in the presence of low levels of nitrate (Wang *et al.*, 1999). The *nap* operon is followed immediately on the chromosome by the *ccm* operon, which encodes the machinery essential for *c*-type cytochrome maturation (Tanapongpipat *et al.*, 1998). The catalytic subunit of Nap, NapA, has also been shown to contain an essential Mo-bis-MGD cofactor and a [4Fe-4S] iron-sulphur centre (Gates *et al.*, 2003). The mechanism of nitrate reduction by Nap is not directly coupled to the generation of Δp in *E. coli* and therefore it is suggested that Nap could fulfil a role in dissipating excess reducing power when growing on very reduced carbon substrates (Brondijk *et al.*, 2004; Sears *et al.*, 1997).

One consequence of the anaerobic, cytoplasmic reduction of nitrate is the production of toxic nitrite, which quickly accumulates in nitrate-respiring bacteria (Cole, 1996). To counter this effect, the regulation of nitrate reductase A is coupled with the regulation of *narK*, which encodes a nitrite efflux protein that actively pumps nitrite out of the cell (Clegg *et al.*, 2002;

DeMoss and Hsu, 1991). Alternatively, *E. coli* can reduce any nitrite present, whether nitrite is encountered as a side-product of nitrate reduction or free in the environment.

The soluble cytoplasmic, NADH-dependent nitrite reductase, NirB

As with nitrate reduction, *E. coli* has evolved two independent enzymes that are capable of the 6-electron reduction of nitrite to ammonia, *nrfA* and *nirB* (shown below).

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O$$

This duplication is not a case of genetic redundancy as the enzymes are differentially regulated, mechanistically distinct and fulfil different physiological roles (Page *et al.*, 1990; Wang and Gunsalus, 2000). The soluble cytoplasmic nitrite reductase, NirB, is a sirohaem-containing enzyme that can catalyse the $6e^-$ reduction of nitrite to ammonia. NirB activity is dependent upon the coordinate oxidation of NADH, which donates the required electrons to NirB. The role of NirB is suggested to be the detoxification of accumulated nitrite during nitrate reduction, due to the coordinate regulation of *nirB* with *narGHI* in high levels of nitrate (Wang and Gunsalus, 2000). The regulation of *nirB* by oxygen and nitrate, via FNR and the two component regulators NarXL and NarQP, will be discussed later in the chapter. Due to the soluble, cytoplasmic nature of NirB, no membrane potential (Δp) is generated by the NADH-dependent reduction of nitrite (Page *et al.*, 1990).

The periplasmic cytochrome-*c* nitrite reductase, NrfA

The second membrane-bound, cytochrome-*c* nitrite reductase, NrfA, couples the reduction of nitrite with the oxidation of formate. The catalytic subunit of the Nrf reductase, NrfA, is dependent upon the coordination of 5 covalently-bound haem groups (Eaves *et al.*, 1998). The Nrf enzyme complex consists of a periplasmic facing, pentahaem *c*-type cytochrome (NrfA) clustered with a periplasmic pentahaem cytochrome (NrfB), a periplasmic iron-sulphur protein containing 4 [4Fe-4S] centres (NrfC) and a membrane-bound quinol dehydrogenase (NrfD) (Hussain *et al.*, 1994). The reduction of nitrite by Nrf and the coordinate

oxidation of formate via the quinol pool leads to the generation of a proton gradient, conserving energy.

Nitric oxide formation and nitrosative stress

The production of endogenous nitric oxide in *E. coli* is generally attributed to the activity of both nitrite reductases, NirB and NrfA (Corker and Poole, 2003; Weiss, 2006). Nitric oxide is generated by the 1-electron reduction of nitrite according to the equation: NO_2^- + $2H^+$ + $1e^-$ → $NO^\cdot$ + H_2O. *E. coli* may also encounter nitric oxide produced by other gastrointestinal bacteria as well as nitric oxide produced chemically from nitrite in an acidified environment (Benjamin *et al.*, 1994; McKnight *et al.*, 1997). The systems used by *E. coli* to remove or detoxify NO may also represent significant virulence factors in pathogenic strains as nitric oxide is also a key bactericidal molecule generated by macrophages to kill bacteria (reviewed by Fang, 2004).

Nitric oxide is highly reactive and will react with other chemicals present in the cell to make secondary toxic intermediates as well as with metal centres, including iron, to form nitrosyl adducts. The iron-sulphur centres of metabolic enzymes, such as aconitase B and fumarase A, and iron-dependent transcriptional regulators such as FNR and Fur, can be de-activated by nitric oxide by the formation of iron-nitrosyl adducts (Cruz-Ramos *et al.*, 2002; D'Autreaux *et al.*, 2004; Justino *et al.*, 2007; Kennedy *et al.*, 1997). Iron-sulphur centres involved in electron transport in respiratory chains seem unaffected by NO and it is possible that NO can bind only to iron-sulphur centres that are accessible at the active sites of enzymes with substrate channels.

The related reactive nitrogen species NO, NO^- and NO^+, all have unique chemistries and react differently in the cell (Hughes, 1999). Nitrosation of cellular components such as nucleotides and thiols involves the transfer of the nitrosyl cation, NO^+. The nitrosyl cation is generated from NO in the presence of a metal ion or oxygen and can be transferred to a

nucleophile with a lone pair of electrons, for instance sulphur or nitrogen. Nitric oxide cannot nitrosate thiols directly in the absence of either oxygen or a metal ion (Pullan *et al.*, 2007). Glutathione present in the cell can become nitrosated under specific conditions and will itself trans-nitrosate many cell components including thiols. Indeed, S-nitrosoglutathione or GSNO is often used as a source of nitrosative stress in investigations. However, the biological effects of these two compounds are likely to be different. Some evidence has been presented that shows decomposition of S-nitrosothiols, in the presence of thiols, leads to the generation of hydroxylamine and nitrous oxide (Arnelle and Stamler, 1995). This is particularly interesting as the source of cytotoxic hydroxylamine is not well understood and hydroxylamine produced enzymatically by the nitrite reductases, NirB and NrfA, is not thought to escape the active site before being reduced to ammonia (Jackson *et al.*, 1981). Nitric oxide can also react with superoxide radicals to produce the highly reactive and toxic product peroxynitrite ($ONOO^-$) (Hughes, 1999). As NO and superoxide are made concomitantly by the innate immune response, the toxicity of peroxynitrite and the ability of bacteria to deal with it may be relevant to the pathogenicity of *E. coli* and other related species (Fang, 2004).

Nitric oxide can also indirectly damage DNA and has been shown to be a potent mutagen in *E. coli* during nitrate metabolism (Weiss, 2006). During nitrosative stress conditions, it is thought that an unidentified endogenous DNA-methylating species, most likely a nitrosamine, is produced, leading to DNA damage (Taverna and Sedgwick, 1996). Endogenous N-nitrosamines have been shown to be produced from secondary amines and nitrite by resting *E. coli* B cells (Kunisaki and Hayashi, 1979). N-nitrosamines have also been shown to be potent DNA-methylating agents and a cause of increased carcinogenesis in rats (Swann and Magee, 1968). It is most likely that N-nitrosamines are the endogenous methylating species that cause DNA-damage when *E. coli* are exposed to nitrosative stress.

The complexity of nitric oxide chemistry and the many toxic secondary reactive nitrogen species made from it (summarised in fig 1.3) mean that it is likely that *E. coli* would

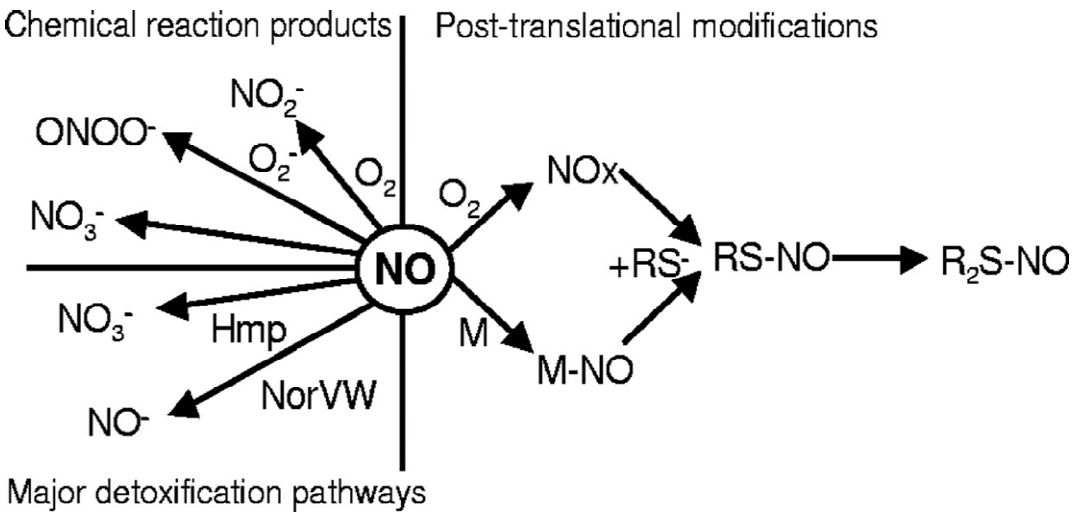

Figure 1.3 Targets of NO and its fate. Taken from Pullan *et al.* (2007)

(Left panel) Unreacted NO appears primarily as nitrite after oxidation or as peroxynitrite after reaction with superoxide. (Lower left panel) The detoxification mechanisms employed by enterobacteria involve primarily the aerobic conversion to nitrate by flavohemoglobin (Hmp) and the one-electron reduction to the nitroxyl anion (NO^-) catalyzed by flavorubredoxin (NorVW). (Right panel) The redox requirements for S nitrosation of thiols (RS^-) are met by transition metals (M) or O_2. Transnitrosation (i.e., transfer of the NO group to R_2S) is directed by nitrosation motifs and/or protein hydrophobic environments

need different enzymes and pathways to deal with and repair the diverse damage to many cellular components that would be caused by these reactive nitrogen species, as well as with the reactive nitrogen species themselves.

Nitric oxide detoxification in *E. coli* by the flavohaemoglobin, HmpA

To counter the toxicity of nitric oxide and related nitrosative stresses, *E. coli* has developed a number of systems that can reduce or detoxify nitric oxide or repair the damage caused by its action. In most cases, the synthesis of these systems is regulated by nitric oxide itself or by the presence of metabolic precursors such as nitrate (Browning *et al.*, 2006; Poole *et al.*, 1996; Tucker *et al.*, 2005). Several systems for reducing or detoxifying nitric oxide have been characterised in *E. coli*. However, how the bacterium repairs damage caused by nitric oxide is less well understood.

The three dimensional structure of HmpA has been resolved and characterised by X-ray crystallography. HmpA belongs to a family of ancient flavohaemoglobins that are well represented in both prokaryotic and eukaryotic phyla (Poole and Hughes, 2000). These proteins usually consist of an N-terminal haem-binding domain associated with a flavin-binding reductase domain, which together are functionally active (Hernandez-Urzua *et al.*, 2003). HmpA was first implicated as a nitric oxide protection system when it was noticed that expression of HmpA was greatly increased in the presence of nitric oxide (Poole *et al.*, 1996), an implication that was later confirmed by the increased sensitivity of a *hmpA* mutant strain of *Salmonella enterica* to a nitric-oxide releasing reagent (Stevanin *et al.*, 2002). Aerobically, HmpA is able to convert nitric oxide to nitrate through dioxygenase activity (Hernandez-Urzua *et al.*, 2003). HmpA is also able to reduce nitric oxide, in the absence of oxygen, to nitrous oxide (N_2O) (Corker and Poole, 2003; Poole, 2005). However, the reaction rate of this reduction is very low and is unlikely to protect the bacterium from endogenously created NO during nitrite metabolism (Gardner and Gardner, 2002).

The *hmpA* promoter is repressed by active FNR, which binds over the -10 component of the *hmpA* promoter and inhibits RNAP binding (Poole *et al.*, 1996). As described in later sections, FNR is de-activated by the presence of oxygen that degrades the co-ordinate iron-sulphur centres of the FNR dimer. Through investigation of *hmpA* expression, a novel mechanism for FNR regulation by nitrosative stress was demonstrated. In this case the iron-sulphur centres of the FNR dimer are attacked by nitric oxide, which leads to the de-repression of *hmpA* in response to the nitric oxide signal (Cruz-Ramos *et al.*, 2002).

The methionine biosynthesis regulator, MetR, has been implicated in the regulation of *hmpA* when the reactive nitrogen species S-nitrosoglutathione is present in the environment demonstrating a further level of complexity in the regulation of these stress responses. MetR binds to two operators in the *hmpA* promoter region and, when occupying both sites, represses transcription. The mechanism leading to the activation of MetR is thought to occur via the nitrosation of homocysteine (Hcy), the MetR cofactor. This leads to a depletion of Hcy and decreased levels of activated MetR resulting in the binding of MetR to only one of the operator sites, which is able to then activate *hmpA* (Membrillo-Hernandez *et al.*, 1998). Furthermore, the *hmpA* promoter region has been identified as containing a functional NsrR-binding motif and has been shown to be under the control of NsrR, the regulator of nitrosative stress, by microarray analysis (Bodenmiller and Spiro, 2006; Filenko *et al.*, 2007; Rodionov *et al.*, 2005).

Nitric oxide reduction by the flavorubredoxin and associated flavo-protein, NorVW

An alternative nitric oxide protection system, a dedicated nitric oxide reductase (NOR), has also been identified in *E. coli.* This protein belongs to the A-type flavoprotein family which is widespread amongst prokaryotes. In contrast to the nitric oxide reductases of denitrifying bacteria, which contain haem-copper ligands, the NOR of *E. coli* has been shown to contain a non-haem di-iron active ligand (Gomes *et al.*, 2002). The nitric oxide reduction system consists of the flavorubredoxin NorV and an associated flavoprotein NorW, which together are able to

detoxify nitric oxide in anaerobic conditions through NADH-dependent NO reductase activity (D'Autreaux *et al.*, 2005). The protein is able to reduce nitric oxide to nitrous oxide anaerobically at a much higher rate than that of HmpA (Gomes *et al.*, 2002), making NorVW the most likely candidate for detoxifying nitric oxide created from nitrite metabolism.

Expression of the flavorubredoxin and its associated flavoprotein, NorVW, has been shown to be absolutely dependent on the function of a novel nitric oxide responsive regulator, NorR, which is expressed divergently from the *norVW* promoter (Hutchings *et al.*, 2002). The expression of *norVW* is co-dependent on the NorR activator and sigma 54 (σ^{54}). Transcription is totally abolished by mutation of *norR* (Gardner *et al.*, 2003) showing an absolute requirement for NorR at the *norVW* promoter. Active NorR, which binds to three recognition sequences in the promoter region of *norVW*, is required to initiate transcription and has led to the proposal that active NorR functions as a trimer (Justino *et al.*, 2005a). NorR was viewed as a unique transcriptional regulator in *E. coli* for its ability to respond directly to the inducer molecule, nitric oxide. However, as mentioned, NsrR may also represent an NO-sensitive transcription factor (Bodenmiller and Spiro, 2006; D'Autreaux *et al.*, 2005).

NorR is a three domain protein that contains a C-terminal helix-turn-helix DNA-binding domain, an N-terminal iron-containing GAF domain and a central AAA+ domain, which exhibits ATPase activity. NorR has been shown to respond to the presence of NO via the non-haem mononuclear iron containing GAF domain, which reversibly binds nitric oxide and changes the conformation of the regulator. This allows for ATPase activity from the AAA+ domain and results in the isomerisation of σ^{54} and transcription activation (D'Autreaux *et al.*, 2005). This mechanism represents an unprecedented mechanism of NO-sensing via a mono-nitrosyl iron interaction (D'Autreaux *et al.*, 2005). The mechanism of NorR activation allows for expression of nitric oxide detoxification machinery in direct response to the presence of toxic nitric oxide without regulatory intermediates.

Nitric oxide reduction by the nitrite reductase, NrfA: an auxiliary role in detoxification

Previously in this chapter, the *c*-type cytochrome NrfA has been described as a nitrite reductase that reduces nitrite to ammonia and produces the toxic side product nitric oxide during anaerobic respiration. However, *in vitro* studies have shown that the Nrf protein can also reduce nitric oxide at a higher rate at the same active site for nitrite reduction (Poock *et al.*, 2002; van Wonderen *et al.*, 2008). The physiological role of NrfA in nitric oxide reduction has also been shown to be significant *in vivo*, by assessing the sensitivity of *nrf* mutant strains to NO (Poock *et al.*, 2002). This study also assessed the reduction of nitric oxide directly by using an NO-specific electrode, and showed that *nrfA* contributes significantly to the rate of reduction of NO in intact cells (Poock *et al.*, 2002). The authors postulated from this information and the manner in which *nrfA* is regulated, that NrfA could afford some respiratory protection from NO in oxygen-limited environments (Poock *et al.*, 2002).

The roles that NrfA, as well as HmpA and NorVW, play in nitric oxide detoxification are not yet fully understood. However, it is unlikely that these three systems are redundant and are most likely to fulfil distinct roles dependent on the exact environment the bacterium is in at any given time.

The *E. coli* hybrid cluster protein, HCP

The hybrid cluster protein of *E. coli* is highly expressed in cells growing anaerobically in the presence of nitrate or nitrite (Filenko *et al.*, 2005; van den Berg *et al.*, 2000). In *E. coli,* the gene encoding Hcp is co-expressed as a two gene operon with the NADH-dependent Hcp reductase, Hcr, in contrast with obligate anaerobic bacteria where *hcr* is absent (van den Berg *et al.*, 2000). Due to the presence of Hcp in cells growing anaerobically on nitrate or nitrite, a role in reactive nitrogen processing has been proposed (Wolfe *et al.*, 2002). In *E. coli*, the expression of Hcp is dependent on FNR, which binds to a site centered at position -72.5 with respect to the transcription start site. Initial investigations into the regulation of the *hcp*

promoter suggested that NarL and NarP enhanced the expression of the promoter in response to nitrate and nitrite. *In vitro* binding of NarL and NarP proteins to a site centered around -72.5 with respect to the transcription start site was demonstrated (Filenko *et al.*, 2005). However, recent unpublished data has shown that the apparent nitrate and nitrite regulation of p*hcp* was actually dependent upon de-repression of NsrR, which binds to a site overlapping the -35 promoter element, repressing transcription, and was independent of NarL or NarP (Filenko *et al.*, 2007)(Dave Chismon, unpublished results).

The hybrid cluster protein, previously known as the prismane protein, was first identified in the obligate anaerobic sulphate reducing bacteria, *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris,* and a crystal structure for these proteins was determined (Moura *et al.*, 1992; Stokkermans *et al.*, 1992). The hybrid cluster proteins of the *Desulfovibrio* species were shown to contain two iron-sulfur clusters, one a standard [4Fe-4S] cubane cluster and the other an unusual [4Fe-2S-2O] cluster or 'hybrid cluster' (Stokkermans *et al.*, 1992) (fig. 1.4). The hybrid-cluster protein of *E. coli* has been shown to contain the same [4Fe-2O-2S] cluster but only a [2Fe-2S] cluster in place of the cubane cluster (van den Berg *et al.*, 2000). The Hcr protein expressed in *E. coli* was also shown to contain FAD and a [2Fe-2S] cluster as cofactors (van den Berg *et al.*, 2000). In *Salmonella enterica,* the expression of *hcp* was shown to be up-regulated in bacteria extracted from activated macrophages, suggesting that in this species HCP may be involved in nitrosative stress management (Kim *et al.*, 2003). Finally, an *in vitro* study, using purified Hcp, showed that Hcp can reduce hydroxylamine to ammonia in a 1:1 ratio with a K_m for hydroxylamine of ~25 mM at pH 7 and ~5 mM at pH 9 (Wolfe *et al.*, 2002). Due to the apparent low affinity of Hcp for hydroxylamine, the physiological relevance of the hydroxylamine reductase activity of Hcp *in vivo* is still in question and was investigated during this project.

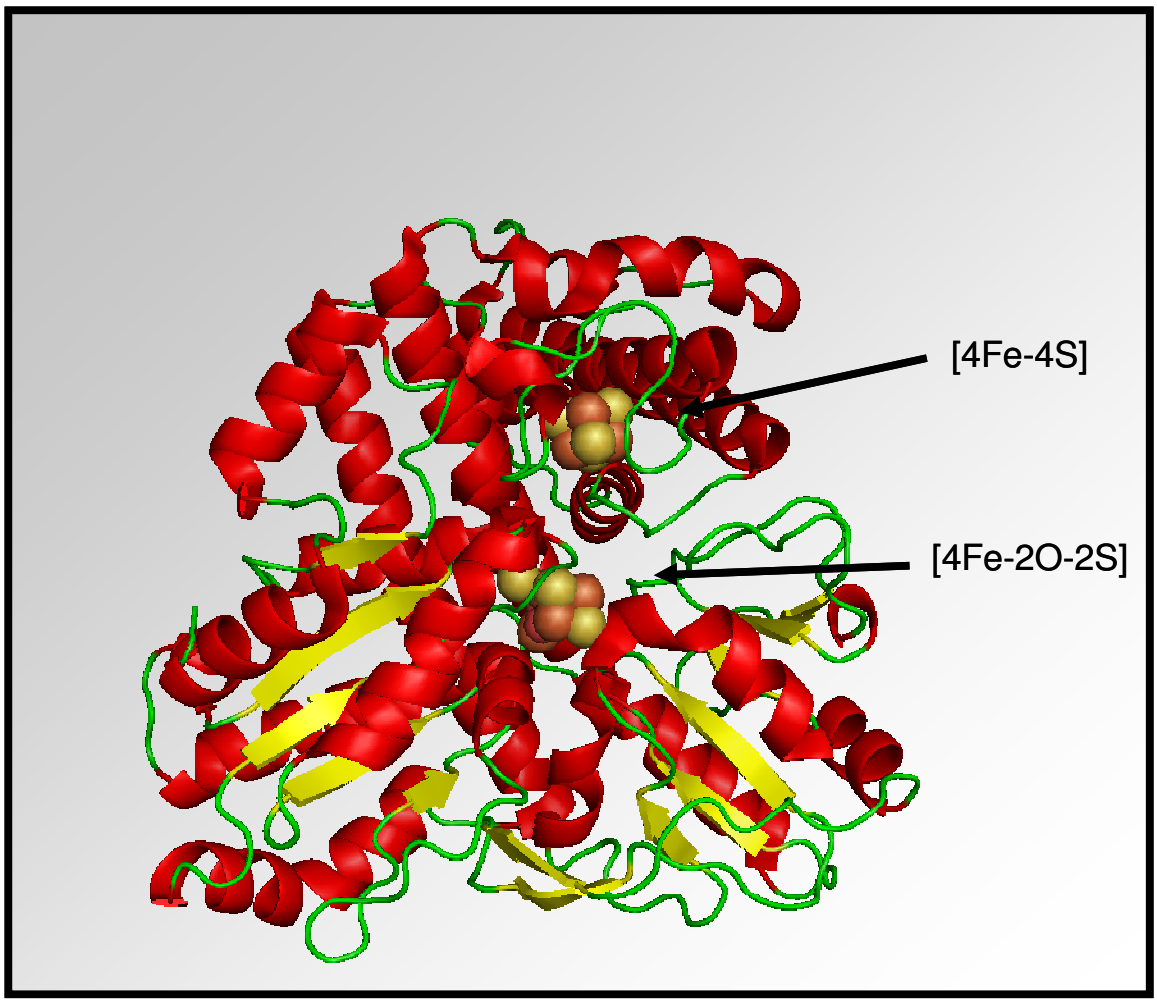

Figure 1.4 The structure of the hybrid cluster protein

The structure of the hybrid cluster protein purified from *Desulphovibrio vulgaris*. Both the coordinated cubane [4Fe-4S] and hybrid [4Fe-2O-2S] iron-sulphur centres are visible and labelled. Protein structural coordinates, accession number, 1E1D, were downloaded from RSCB protein data base and modelled using PyMOL modelling software.

The bacterial RNA polymerase

At every stage of production, the synthesis of protein from DNA can be regulated. However, for reasons of energy economy, the key step to be regulated is transcription (Browning and Busby, 2004). The key player in transcription of DNA to RNA in *E. coli* is the multiple subunit, DNA-dependent RNA polymerase (Browning and Busby, 2004). The core enzyme of RNA polymerase (RNAP) consists of two α-subunits, the large β and β' subunits and a small subunit, ω, which is not essential for transcription initiation but is thought to bind β' and assists in RNAP assembly (Darst *et al.*, 1989; Minakhin *et al.*, 2001). The core enzyme is competent for RNA synthesis from DNA but is unable to initiate transcription from promoters. In order for RNAP to recognise and bind the promoter, an additional subunit or σ factor must associate with the core-RNA polymerase to make the RNAP holo-enzyme (Burgess *et al.*, 1969; Ishihama *et al.*, 1973). The chromosome of *E. coli* encodes seven different σ factors that are functional under certain conditions dependent upon the environment that the bacterium is in (Gross *et al.*, 1998; Ishihama, 1999). All *E. coli* sigma factors share common features with the exception of the sigma factor, σ^{54} (Merrick, 1993). The holo-enzyme is then able to recognise and bind to specific target DNA sequences and initiate transcription. *E. coli* encodes seven different sigma factors that compete for the RNAP core enzyme in response to different stimuli (Gruber and Gross, 2003). The major sigma factor or 'house-keeping' sigma factor is σ^{70}, also referred to as σ^{D}, encoded by the *rpoD* gene. Sigma 70 regulates expression of around 1000 transcrpition units in *E. coli* (Ishihama *et al.*, 1973). Each sigma factor recognises a different promoter sequence and activates transcription of different sets of genes. The four specific promoter elements that allow for promoter recognition by σ^{70} RNAP-holoenzyme have been identified and include: the -10 and -35 elements, an extended -10 sequence and an UP-element. The -10 and -35 elements, which are positioned 10 and 35 nucleotides upstream of the transcription start site, are recognised by domain 2 and 4 of the

RNAP σ factor, respectively (Campbell *et al.*, 2002). The consensus sequence of the -10 and -35 elements have been determined and are TATAAT and TTGACA, respectively. The extended -10 sequence, which is found upstream of the -10 motif, consists of a TG doublet at positions -13 and -14 that is recognised by domain 3 of the σ factor (Barne *et al.*, 1997). The fourth promoter element or UP-element, consisting of an AT-rich region of approximately 20 bp, is found upstream of the -35 element and is recognised by the α-carboxy-terminal domain of the RNA polymerase enzyme (Gourse *et al.*, 2000).

The central dogma of transcription regulation is that the supply of RNA polymerase is limited. Many of the RNA polymerase complexes present in the cell are used continuously for the transcription of stable RNAs, meaning that the amount of RNAP free for transcription of the 4000-5000 protein-coding genes in *E. coli* is severely limited. For this reason the action of RNAP and the genes that it transcribes must be prioritised and directed to ensure that genes are expressed at the right time in response to a changing environment. Several factors regulate the distribution of RNA polymerase across the chromosome; these include promoter DNA sequences, chromosomal folding, competition for σ factors, small ligands and transcription factors (reviewed by Browning and Busby, 2004).

Transcription factors

Transcription factors are sequence-specific DNA-binding proteins that control the expression from target promoters. In most cases, transcription factors respond to an environmental signal that changes their DNA-binding affinity, allowing them to bind to specific target sequences and up- or down-regulate transcription (Browning and Busby, 2004). To date, around 300 transcription factors have been found to be encoded on the *E. coli* chromosome (Perez-Rueda and Collado-Vides, 2000). Many of these transcription factors regulate transcription at only one target promoter, while others have been found to alter the gene expression profile of a vast number of genes and are therefore referred to as global

transcription factors. Many *E. coli* transcription factors can function as both activators and repressors depending on the position at which they bind in the promoter region. Activation can occur by recruitment of the RNAP to the promoter region or by facilitating open complex formation. Alternatively repression can occur by several mechanisms including steric hindrance of RNAP, binding over or proximal to the promoter, DNA looping or repression by interfering with the interaction between RNAP and an activator, sometimes referred to as anti-activation (Browning and Busby, 2004).

Transcription activation

Simple activation occurs at promoters by three distinct mechanisms (Browning and Busby, 2004). In Class I-activated promoters, an activator binds to a specific target sequence upstream of the -35 promoter element and the UP-element. Due to the flexible nature of the linker region of the protein that joins the α-carboxy-terminal domain and the α- amino-terminal domain of the RNAP, class I activators can bind at several distances with relation to the transcription start site. When bound in this configuration, the transcription activator can make contact with the RNAP enzyme. At class II activated promoters, the transcription factor binds to a specific target sequence, upstream of the -35 element but downstream of the UP-element or α-CTD binding sequence. In this case several contacts can be made between the transcription factor and the RNA polymerase. At class III dependent promoters, two or more transcription factors are required to bind to the promoter region and make several contacts with RNA polymerase (Busby and Savery, 2007). The best understood *E. coli* transcription factors are the cyclic-AMP receptor protein (CRP) and it homologue, the regulator of fumarate and nitrate reduction FNR. When FNR functions as a class I activator, it typically binds to a target sequence upstream of the -35 promoter element. When bound in this position, FNR makes specific protein-protein contacts with the proximally-bound α-CTD of RNAP (Barnard *et al.*, 2004; Lee *et al.*, 2000; Williams *et al.*, 1997). The region of FNR that makes contact with the

α-CTD of RNAP polymerase is termed activating region 1 (AR1) and is essential for transcription activation. At class II, FNR dependent promoters, FNR typically binds to a site centred at -41.5 with respect to the transcription start site. When bound at this position, FNR makes three specific protein-protein interactions with the proximally-bound RNA polymerase. The upstream half of the FNR dimer makes contact with the upstream-bound α-CTD of RNAP at AR 1 (fig 1.5) Two specific contacts are made between the downstream subunit of the FNR dimer and RNAP with AR2 of FNR contacting the α-NTD of RNAP and AR3 contacting domain 4 of the RNAP associated σ factor (fig. 1.5) (Li *et al.*, 1998). The locations of activating regions 1, 2 and 3 on the FNR protein are similar to those found in the homologue, CRP. However, activating region 2 of FNR is not essential for class II activation by FNR and conversely, activating region 3 of CRP is not required for CRP-dependent class II activation (Li *et al.*, 1998).

The regulation of anaerobic respiration by FNR

The transition from aerobic to anaerobic respiration is regulated at many operons by the global transcription factor FNR. No crystal structure of FNR has been published to date. The amino acid sequence of FNR has been shown to have considerable similarity with CRP, exhibiting a very similar C-terminus that contains a helix-turn-helix motif that directly contacts DNA in CRP (Shaw *et al.*, 1983). However, the two proteins differ at the N-terminus, as FNR contains a cysteine rich N-terminal extension of which four cysteine residues are vital to its function (Green *et al.*, 1993). The role of the cysteine residues, Cys^{20}; Cys^{23}; Cys^{29} and Cys^{122}, is to co-ordinate an iron-sulphur centre at the N-terminus of the FNR monomer that facilitates the response to oxygen levels (Crack *et al.*, 2008a). The iron-sulphur centre of FNR exists predominantly in two forms, namely a reduced $[4Fe\text{-}4S]^{2+}$ in the active dimeric form of the protein, and an oxidised $[2Fe\text{-}2S]^{2+}$ in the inactive monomeric form (fig 1.6). According to this information it has been suggested that equilibrium between the two forms exists, dependent on the oxygen concentration in the environment (Kiley and Beinert, 2003). The inter-conversion

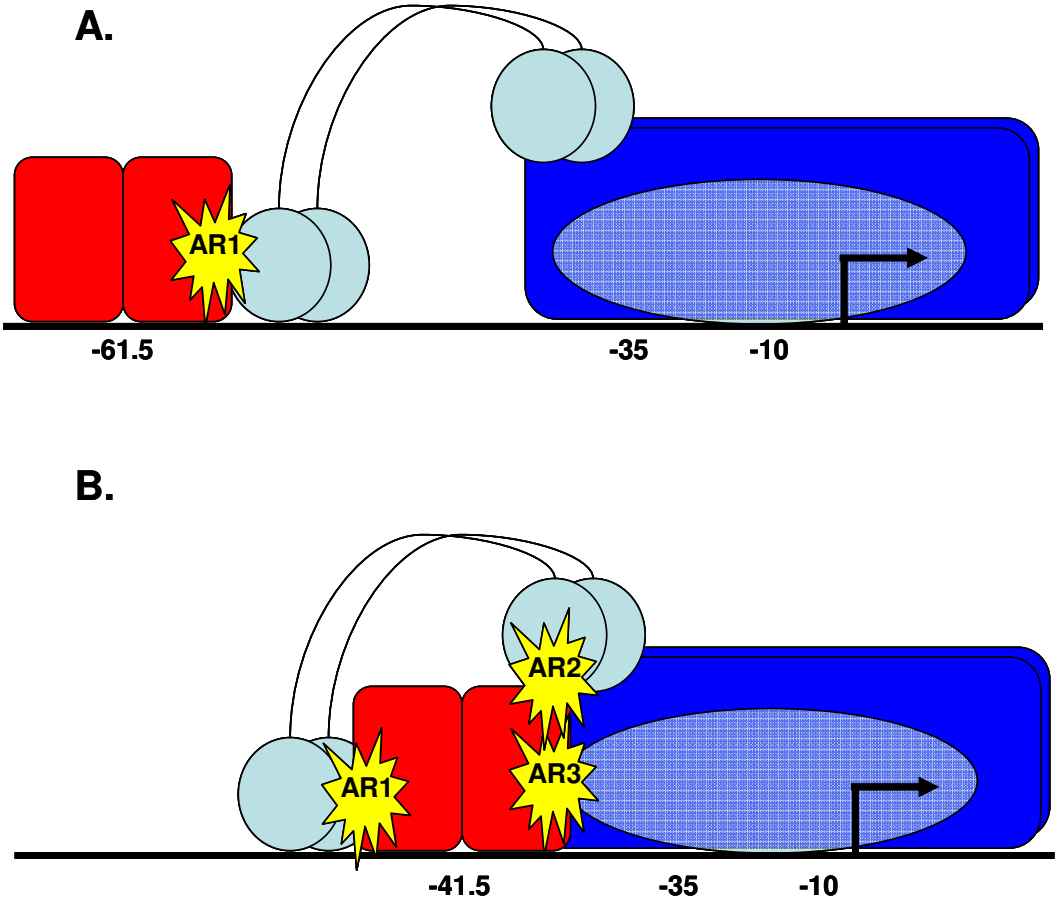

Figure 1.5 Class I and Class II transcription activation by FNR in *Escherichia coli*

A. Class I activation. FNR binds to an upstream site, typically centered at position -61.5 and contacts the αCTD of RNA polymerase via AR1, activating transcription.

B. Class II activation. FNR binds to a target proximal to the promoter –35 element and the bound activator interacts with the αCTD of RNA polymerase via AR1, the αNTD of RNA polymerase via AR2 and domain 4 of σ^{70} via activating region 3. The FNR dimer typically binds at a position centered at position -41.5.

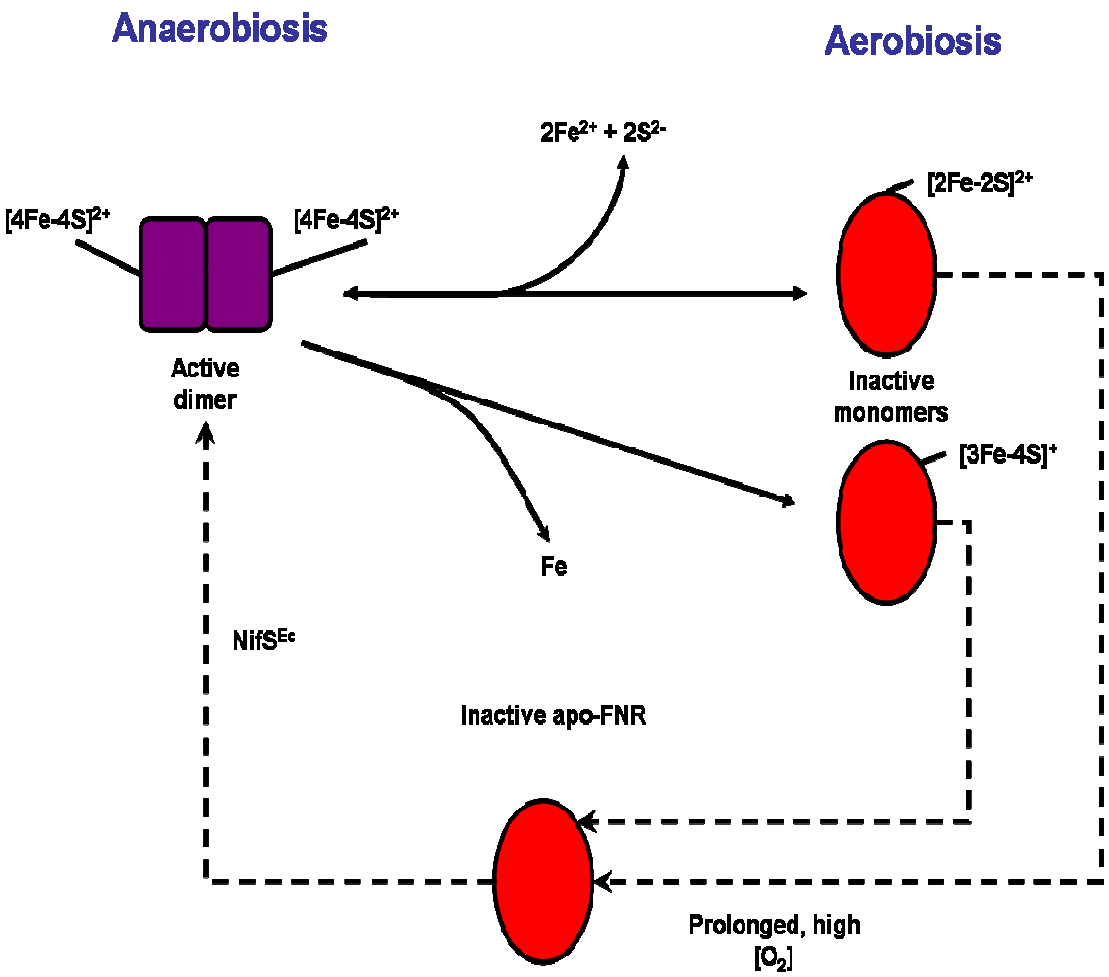

Fig. 1.6 Oxygen-sensing by FNR

In the presence of oxygen, FNR exists as an inactive monomer that is unable to bind DNA. When oxygen is depleted, the coordinated iron-sulphur centre of FNR is reduced to a [4Fe-4S] centre causing a change in conformation, allowing for protein dimerisation. As a dimer, FNR is able to bind DNA at specific target sequences and activate or repress transcription. During prolonged exposure to oxygen, the coordinated iron-sulphur centre can become completely degraded leading to inactive apo-FNR.

between the $[4Fe\text{-}4S]^{2+}$ state and the $[2Fe\text{-}2S]^{2+}$ cluster occurs in two stages via a $[3Fe\text{-}3S]^{1+}$ intermediate, the first being a rapid oxygen-dependent step, the second a slower oxygen-independent step (Crack *et al.*, 2008a). Active FNR can also act as a repressor, switching off genes involved in aerobic respiration or fermentation as well as auto-repressing the *fnr* gene (Spiro and Guest, 1990).

Some significant evidence has also shown that the function of FNR can be negated by the binding of nitric oxide to the coordinate iron-sulphur centre as an S-nitroso adduct (Crack *et al.*, 2008b; Cruz-Ramos *et al.*, 2002). FNR has been shown to repress the transcription of the gene encoding the NO detoxifying flavohaemoglobin, HmpA, by binding to a position centred around +5.5 in the *hmp* promoter, and this binding can be overcome with the addition of NO (Cruz-Ramos *et al.*, 2002). The sensitivity of FNR to NO as well as oxygen has far-reaching implications for global gene regulation in response to nitrosative stress.

The dual-acting, two-component regulators, NarXL and NarQP

Many of the genes involved in anaerobic nitrate and nitrite respiration are co-regulated by FNR and two similar two-component regulator systems, NarQP and NarXL, that respond to the presence of nitrate and nitrite (Stewart, 1993). The Nar two-component systems conform to the model of a transmembrane histidine protein kinase (NarX/ Q) paired with a cytoplasmic response regulator (NarL/ P). In both cases, the histidine protein kinase (HPK) component auto-phosphorylates at a conserved histidine residue in response to an environmental signal, such as nitrate, expending ATP in the process (Cavicchioli *et al.*, 1995). The HPK domain is then de-phosphorylated by the response regulator and the phosphate group is passed from the conserved histidine of the HPK to a conserved aspartate on the response regulator (Egan and Stewart, 1991). The phosphorylated active form of the response regulator is then able to bind DNA at specific target sites and alter expression from those promoters (fig. 1.7).

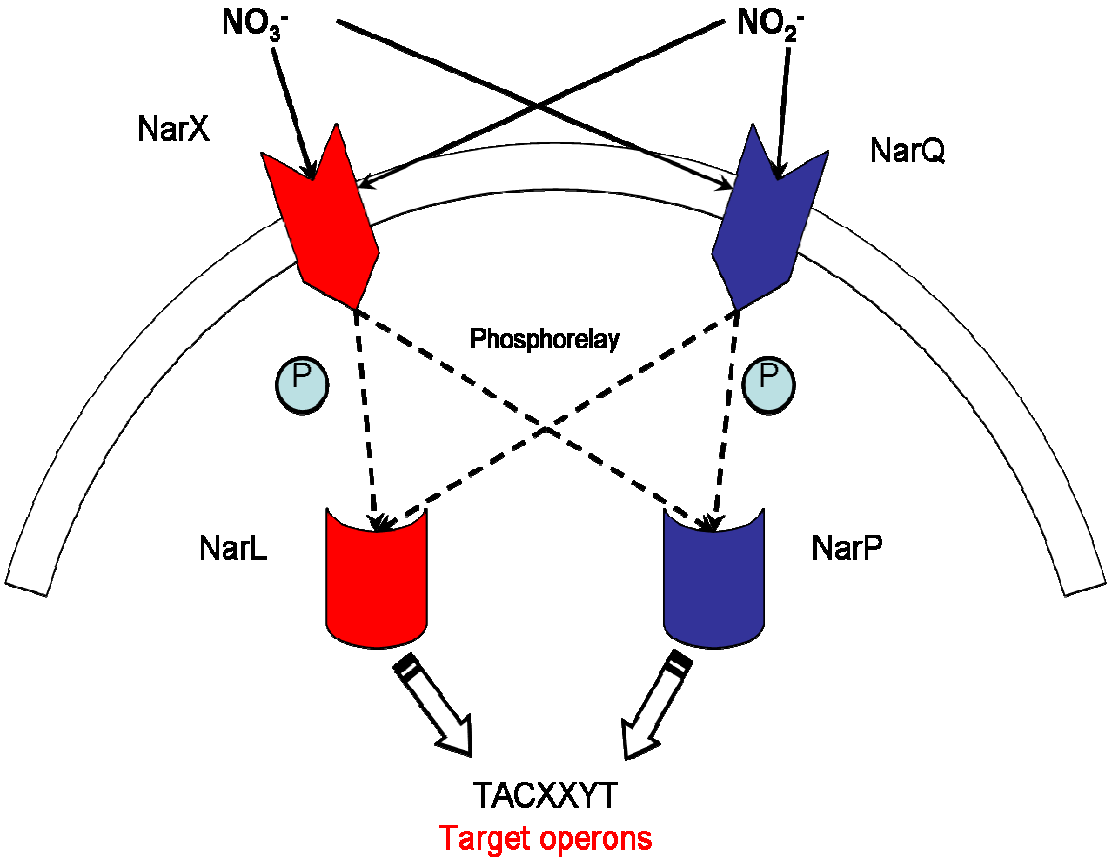

Figure 1.7 The dual-acting two-component regulators NarXL and NarQP

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

The sensor kinases, NarX and NarQ, show 32% identity and 68% similarity to each other with most of the highly conserved regions located in the cytoplasmic domains (Cavicchioli *et al.*, 1995). The periplasmic domains of the two sensor kinases are relatively dissimilar with the exception of a conserved, 17 amino acid region designated the P-box, which is almost identical in the two sensors (Cavicchioli *et al.*, 1996). The P-box domain is essential to protein function and is proposed to detect nitrate and nitrite in the periplasm and modulate signal transduction to the response regulator through the cytoplasmic domain (Cavicchioli *et al.*, 1996). Both NarX and NarQ contain two conserved histidines and one conserved asparagine, His^{399}, His^{513} and Asn^{509} in NarX; His^{370}, His^{484} and Asn^{480} in NarQ, which are vital for phosphorylation of NarL (Cavicchioli *et al.*, 1995). Only His^{399} of NarX was necessary for NarL-phoshate dephosphorylation (Cavicchioli *et al.*, 1995). It is proposed that His^{399} and His^{370} of NarX and NarQ, respectively, are the site of autokinase activity in response to nitrate (Cavicchioli *et al.*, 1995).

It is generally accepted that NarX plays a key role in the differential response between nitrate and nitrite. NarX autokinase activity has been shown to be 100 times more sensitive to the presence of nitrate than nitrite (Lee *et al.*, 1999). In response to the presence of nitrate, NarX phosphorylates both NarL and NarP but in response to nitrite, NarX phosphorylates NarP while acting as a NarL phosphatase (Williams and Stewart, 1997). This leads to a differential gene expression in response to nitrate and nitrite, synthesising the enzymes best suited to utilise the available oxidants (Goh *et al.*, 2005; Unden and Bongaerts, 1997).

The response regulators, NarL and NarP are both 24 kDa proteins that share 44% identity and 64% similarity in their amino acid sequence. In response to nitrate in the periplasm, NarL and NarP can be phosphorylated by either NarX or NarQ and, once phosphorylated, can bind to DNA at specific target sequences (Egan and Stewart, 1991). NarL and NarP both contain a conserved aspartate residue, Asp^{59} in both proteins, which must be phosphorylated for the protein to bind DNA *in vivo* (Egan and Stewart, 1991). The solution of

the crystal structure of the response regulator, NarL, revealed that the tertiary structure of the protein consisted of the N-terminal receiver domain, the site of phosphorylation, which is folded into a five-strand β sheet flanked by five helices similar to that seen in the chemotaxis protein CheY (Baikalov *et al.*, 1996). The C-terminal DNA-binding domain of the protein consisted of four compacted α-helices, two of which formed a helix-turn-helix motif, typical of DNA-binding proteins (Baikalov *et al.*, 1996). The two functional domains are linked by a 13-residue flexible tether that is not visible in the crystal structure. In the crystal structure, which resolved the non-phosphorylated form of the protein, the C-terminal domain is rotated against the N-terminal receiver domain in such a way that DNA binding would be negated (Baikalov *et al.*, 1996). Investigations into changes in the NarL protein structure caused by phosphorylation indicated that when the conserved aspartate residue is phosphorylated, it triggers a change in conformation of the protein and a domain separation that allows for DNA binding at target promoters (Zhang *et al.*, 2003) (fig1.8).

Both regulator proteins (NarP/ L) bind to a heptameric DNA target sequence in the regulatory region of target genes. The heptameric binding sequence for both regulator proteins was shown to be TACXXYT, where X can be T or C, and Y can be A or C (Tyson *et al.*, 1994). However, NarP has been shown to only bind to target sites where the heptameric sequence is arranged in an inverted repeat conformation with a two bp spacer, also referred to as the 7-2-7 conformation, whereas NarL will bind to 7-2-7 sequences as well as other arrangements (Darwin *et al.*, 1997).

The differentiation between anions by the sensor kinases, NarX and Q, and the differential binding of NarL and P to target sequences allows *E. coli* to discriminate between oxygen alternatives and synthesise proteins of alternate metabolic pathways to utilize them. At the start of this project, all promoters known to be activated by either NarL or NarP were dependent upon co-activation by FNR.

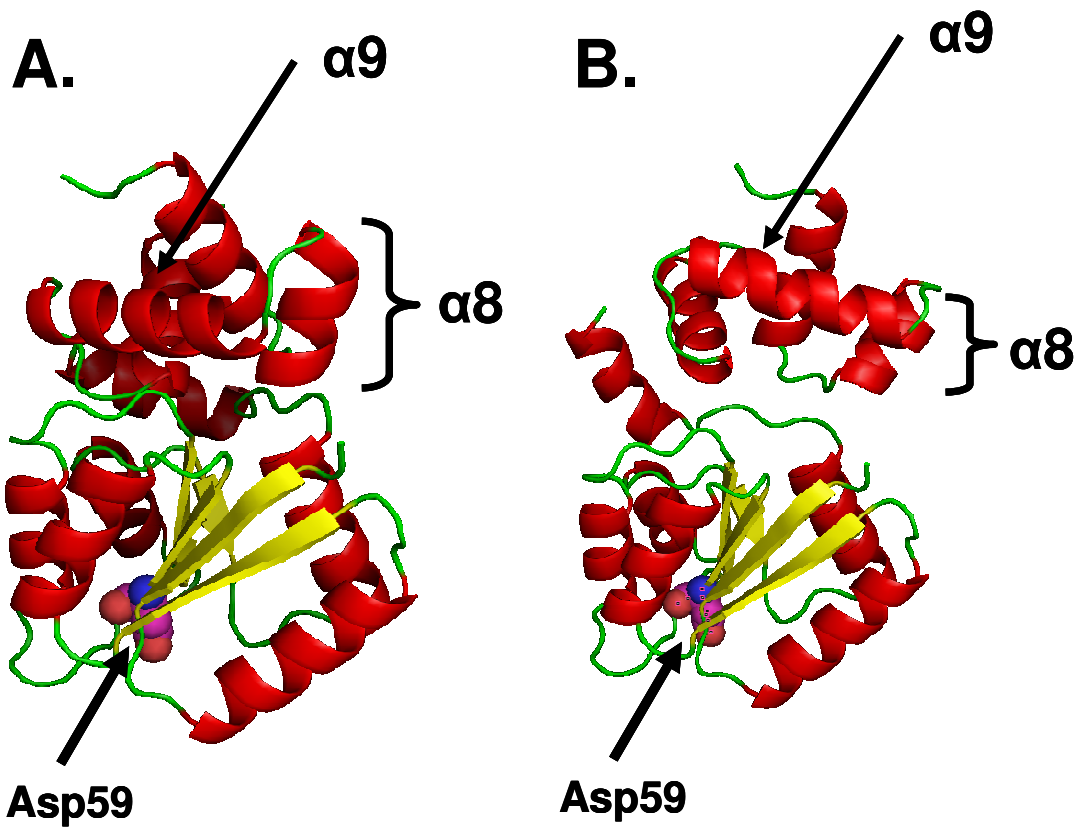

Figure 1.8 Phosphorylation of NarL leads to domain separation that allows DNA binding

A. This figure shows a cartoon ribbon representation of the X-ray crystal structure of NarL (PDB code 1RNL) to a resolution of 2.40 Å. The protein is organised into two distinct domains joined by a flexible linker. The N-terminal receiver domain is folded into a 5-strand *β*-sheet surrounded by 5 α helices and contains the site of protein phosphorylation, Asp59. The C-terminal domain consists of four compacted α helices. In the unphosphorylated form of the protein the C-terminal is rotated against the N-terminal in such a way that DNA binding by the helix-turn-helix motif, formed by α8 and α9, would be inhibited.

B. This figure shows a theoretical structure of the phosphorylated form of NarL. When NarL becomes phosphorylated at Asp59, a domain separation is triggered, most likely through a hinge-bending motion that leaves the distinct N and C-terminal domains in tact. This predicted conformation change was modelled by rotating the *psi* and *phi* bonds of Gly126 in the flexible linker, leading to domain separation. This figure was interpreted from the results of Zhang *et al.* (2003) using PyMOL modelling software.

Regulation of transcription by the nucleoid associated protein, Fis

The small DNA-binding protein, Fis, or the Factor for Inversion Stimulation, is one of a small family of DNA-binding proteins that aid in structuring the compacted *E. coli* chromosome (McLeod and Johnson, 2001). Unlike the eukaryotic equivalents, histones, the nucleoid associated proteins of bacteria form relatively unstable associations with DNA (McLeod and Johnson, 2001). Fis was identified as being an essential stimulus of DNA-inversion catalysed by site specific recombinases in bacteriophage, Mu and P1 (Haffter and Bickle, 1987; Johnson and Simon, 1985). However, Fis has also been shown to have an effect on the structuring of the *E. coli* chromosome, DNA replication and, more recently, transcription regulation (Filutowicz *et al.*, 1992; Ishihama, 1999; McLeod and Johnson, 2001). As with all nucleoid-associated proteins, Fis binds DNA non-specifically at high physiological levels but has been shown to bind preferentially to a core consensus sequence of 15 bp, reading GNtYAaWWWtTRaNC, where Y= C or T, W= A or T, R= G or A and N= any base. (Hengen *et al.*, 1997). Depending upon the DNA sequence flanking the Fis binding site, the protein can contact up to 27 bp of DNA and introduce bends into the DNA structure ranging from less that 50° to greater than 90° (Pan *et al.*, 1996). The activity of Fis is regulated predominantly by the relative abundance of the protein in the cell at any given growth phase (McLeod and Johnson, 2001). Fis levels during stationary phase are negligible but increase to between 25,000 and 40,000 copies per cell during early exponential phase (Ali Azam *et al.*, 1999). Fis levels are also affected by medium composition and are greatest in rich media (Ball *et al.*, 1992). Fis has been shown to have a diverse role in the *E. coli* cell and controls the expression of several genes that encode enzymes required for anaerobic nitrate or nitrite reduction. The regulation ofthese genes will be detailed in later sections.

The regulator of nitrosative stress, NsrR

Further complexity in the regulation of nitric oxide metabolism was uncovered in a recent computational biology study (Rodionov *et al.*, 2005). The authors of this study used comparative sequence analysis of the upstream regions of the *hcp* gene (hybrid cluster protein), a gene known to be regulated by anaerobiosis and nitrate or nitrite. In the sulphur-reducing bacterium, *Desulfovibrio vulgaris,* the regulation of *hcp* is controlled by an FNR/ CRP-like transcription factor, HCR. However, no such regulator exists in *E. coli*. By aligning the promoter regions of the *hcp* gene from several obligate anaerobic bacteria, a conserved palindromic consensus sequence was identified that was shown to be a candidate binding site for an unknown transcription factor (Rodionov *et al.*, 2005). The consensus sequence, gATGyAT-(N_5)-ATrCATc, was also reported in the promoter regions of *hmpA, ytfE,* and *ygbA;* genes that have been confirmed or implicated in nitric oxide stress management (Justino *et al.*, 2005b; Rodionov *et al.*, 2005). In addition to the sites identified in Rodionov *et al* (2005), a potential site has also been identified in the promoter of the operon of unknown function, *yeaR-yoaG,* and a 4-fold, NsrR-dependent repression effect was observed in a microarray analysis of the NsrR regulon (Filenko *et al.*, 2007). It is suggested that the NsrR protein of *E. coli* is directly responsive to nitric oxide (Bodenmiller and Spiro, 2006). NsrR belongs to the Rrf2 family of transcription regulators that includes the iron-sulphur containing transcription regulator, IscR. The mechanism by which NsrR is able to detect nitric oxide has not been determined in *E. coli* but the sequence similarity to IscR and the presence of a number of conserved cysteine residues suggests that an iron-sulphur centre may be coordinated in the protein (Tucker *et al.*, 2008). The NsrR proteins of other bacteria have been purified and analysed and it has been demonstrated that the proteins often coordinate an NO-sensitive iron-sulphur centre of some form. The NsrR protein of the human pathogen, *Neisseria gonorrhoeae,* has been shown to contain an NO-sensitive [2Fe-2S] centre that binds NO, limiting the DNA-binding activity of the protein to target DNA sites *in vitro* (Isabella *et al.*, 2009). Similarly, the

NsrR protein of the Gram-positive bacteria, *Streptomyces coelicolor* and *Bacillus subtilis,* have been shown to contain NO-sensitive [2Fe-2S] and [4Fe-4S] centres, respectively (Tucker *et al.*, 2008; Yukl *et al.*, 2008). When the purified NsrR protein of *Streptomyces coelicolor* is exposed to exogenous nitric oxide the cluster is nitrosylated, resulting in the loss of DNA-binding activity *in vitro* (Tucker *et al.*, 2008). It is likely that NsrR might be a global repressor of nitric oxide metabolism and its role in the regulation of genes that are known to be or implicated in nitric oxide stress management will be investigated during this study.

The regulation of anaerobic respiration

The major nitrate reductase, NarGHI, and the soluble, NADH-dependent nitrite reductase, NirB, are both expressed during anaerobic growth in the presence of high environmental concentrations of nitrate. The promoter of *narGHI* is dependent upon the binding of the global regulator FNR at a position centred around -41.5, where FNR functions as a class II activator, contacting RNA polymerase (Dong *et al.*, 1992). Full activation of p*narGHI* in response to nitrate is dependent upon phospho-NarL. The *narG* promoter region contains up to nine NarL operators arranged in two groups: one proximal to the transcription start site, around -80 and one distal, grouped around -200 (Dong *et al.*, 1992; Li *et al.*, 1994; Walker and DeMoss, 1994). However, only two of these NarL operators are absolutely required for full expression: a single heptamer centered at -195 and a single heptamer centered around -80 (Darwin *et al.*, 1996). The activity of the *narG* promoter has also been shown to be dependent on the binding of the nucleoid-associated protein IHF (integration host factor), which binds in a position between -106 and -144 with respect to the transcription start site, in between the binding site of FNR and NarL. When bound in this position it is proposed that IHF introduces a bend in the DNA, which may bring the upstream bound NarL into closer proximity with the bound FNR (Schroder *et al.*, 1993).

Similarly, the activity from the *nir* promoter is maximal during anaerobic growth in the presence of high concentrations of nitrate. Expression of *nir* requires the binding of FNR to a site centred at -41.5 and the binding of either phosphor-NarL or NarP to a proximal site centred around -69.5 with respect to the transcription start site (Browning *et al.*, 2000; Jayaraman *et al.*, 1989; Tyson *et al.*, 1993). Expression of *nir* has also been shown to be repressed by the nucleoid-associated proteins, FIS and IHF, which bind to sites centred around -142 (FIS I) and +23 (FIS II) and -88 (IHF I) (Browning *et al.*, 2000). More recently a second site for IHF binding, centred around -115 (IHF II) and a third FIS site, centred around -97 (FIS III) have been identified and it was shown that FIS, IHF and FNR can all bind simultaneously to form a multi protein-DNA complex (Browning *et al.*, 2004b). It has been postulated that the binding of NarL and NarP at the *nir* promoter is needed to overcome the repression caused by FIS and IHF, and that observed NarL activation is a result of the relief of the distortion of nucleoid-protein structure, allowing for FNR-dependent transcription activation (Browning *et al.*, 2004b). Finally, *nir* expression is also regulated in response to catabolite availability by the Catabolite Repressor Activator protein, Cra, which represses the *nir* promoter in minimal medium (Tyson *et al.*, 1997). This regulatory network ensures that Nir is synthesised only in rich medium in the presence of high environmental nitrate.

The co-regulation of NarG and NirB by anaerobiosis and high nitrate strongly suggest that both enzymes are part of the same pathway to optimally reduce nitrate to ammonia. When high nitrate concentrations are available in the environment, NarG-dependent nitrate reduction would generate energy through the coupling of reduction with proton translocation, while NirB would reduce the toxic nitrite that would be generated by NarG, but would not conserve energy from nitrite reduction.

The regulation of the periplasmic nitrate reductase, Nap, and the cytochrome-*c* nitrite reductase, Nrf are coordinated in a similar fashion. Both operons are maximally expressed during anaerobic growth in the presence of low levels of nitrate and nitrite. Regulation of the

nap promoter is dependent upon the binding of FNR at a position centered around -62.5, a site typical of class II activated promoters (Darwin *et al.*, 1998; Wang *et al.*, 1999). Maximal expression of *nap* is also dependent upon the binding of phospho-NarP to a 7-2-7 inverted repeat sequence found in an unusual position, -44.5, between the -35 promoter element and the upstream FNR (Darwin *et al.*, 1998). NarP bound at this position is required for maximal promoter expression. The NarP homologue, NarL, is also able to bind to this heptameric inverted repeat sequence but represses transcription when bound at this position, leading some to postulate that NarP and FNR activate transcription synergistically and that NarL antagonizes NarP binding (Darwin *et al.*, 1998). This means that *nap* can only be expressed when very low levels of nitrate or nitrite are available, as high nitrate would induce NarL activity.

The promoter of *nrf* is also active under anaerobic conditions where nitrate and nitrite are available in limited concentration. As with other nitrate and nitrite reductases discussed, *nrf* expression is totally dependent upon FNR, which binds to a position centered at -41.5 with respect to the transcription start site, typical of a class II dependent promoter. The promoter also contains a number of NarL and NarP operator sequences, for which NarL and NarP bind with different affinities (Browning *et al.*, 2005). A 7-2-7 inverted repeat sequence, an operator site for NarL and NarP, is situated upstream of the FNR binding site, centered around -74.5. The promoter region also contains several single heptamers that NarL binds with low affinity (Browning *et al.*, 2006). In the presence of low nitrite or nitrate, NarL and NarP are phosphorylated and bind to the high affinity NarL/ P site in the *nrf* promoter, enhancing expression. However, in high concentrations of nitrate, NarL is phosphorylated and occupies the lower-affinity binding sites, repressing transcription (Wang and Gunsalus, 2000). Regulation at the *nrf* promoter is complicated by the binding of nucleoid-associated proteins, FIS and IHF, to the promoter. Current opinion is that IHF suppresses transcription when bound to a site centered around -54 with respect to the transcription start site, but that the promoter is insulated from the suppression effect of IHF bound at -54 by the binding of NarL or NarP to

the proximal site (Browning *et al.*, 2006). In contrast to the expression of the alternative nitrite reductase, *nir,* expression from the *nrf* promoter is unaffected by Cra (Tyson *et al.*, 1997).

The regulation of *nap* and *nrf,* in response to anaerobiosis and low levels of nitrate, ensures that both enzymes are synthesized concomitantly. It is thought that the purpose of these enzymes is to scavenge any available nitrate and nitrite when it is available in low concentrations in the environment, and coordinate their reduction with the translocation of protons to conserve energy (Potter *et al.*, 2001).

As can be seen in the case of anaerobic nitrate and nitrite reduction, enzymes involved in similar pathways under similar environmental conditions are often regulated by the same transcription factors in response to the same environmental signals. This fact was exploited during this study to investigate the function of genes of unknown function that are regulated under specific growth conditions.

The identification of genes implicated in nitrosative stress management by their regulation

Over the last four years, several micro-array studies have attempted to define the number of genes that are expressed in response to nitric oxide or other compounds known to cause nitrosative stress such as S-nitrosoglutathione and nitrite (Constantinidou *et al.*, 2006; Flatley *et al.*, 2005; Justino *et al.*, 2005b; Mukhopadhyay *et al.*, 2004). Due to the different nature of each of these experiments, there were some discrepancies in the number and identity of the genes shown to be up-regulated. In all five of these independent studies, the expression of three genes, *norV, norW* and *hmpA,* was shown to be consistent. The products of these genes are known to be involved in nitrosative stress management (summarized in table 1.1). The expression of these genes under all conditions in which nitrosative stress can occur suggests a ubiquitous role for these proteins under all conditions tested. The fact that both HmpA and NorVW have been shown conclusively to reduce or oxidize NO under different conditions is consistent with the suggestion that despite the differences in the type of nitrosylative stress

Table 1.1 Genes up-regulated in response to different sources of nitrosative stress in independent microarray studies.

		Up-regulation (√) in response to nitrosative stress				
		Source of nitrosative stress				
	Gene	GSNO	Nitric oxide	Nitrite	Nitrite*	Reference
Group 1	*norV*	√	√	√	√	[1,2,3,4,5]
	norW	√	√	√	√	[1,2,3,4,5]
	hmpA	√	√	√	√	[1,2,3,4,5]
	hcp-hcr	√	√	√	√	[4,5]
	ytfE	√	√	√	√	[2,3,4,5]
	ygbA	√	√	√	√	[3,4,5]
Group 2	*yeaR*		√	√	√	[2,4]
Group 3	*yibIH*			√	√	[4]
	ogt			√	√	[4]

Table 1.1 shows the up-regulation of genes in response to different sources of nitrosylative stress. The column, nitrite*, shows genes that were up-regulated by nitrite in a strain lacking functional FNR, indicating unusual regulation [4]. Genes in group 1 have been shown to be up-regulated in all conditions of nitrosylative stress in two or more independent studies. Genes in group 2 have been shown to be up-regulated in response to pure nitric oxide and the source of endogenous NO, nitrite, by two or more independent studies. Genes in group 3 were shown to be up-regulated only in the presence of nitrite but in an FNR-independent manner. The studies summarized in this table and a brief description of the conditions they tested are:

1. Transcriptional responses of *E. coli* to S-nitrosoglutathione under chemostat conditions (Flatley *et al.*, 2005)
2. Transcriptional responses of *E. coli* to nitric oxide (Justino *et al.*, 2005b)
3. Transcriptional responses of *E. coli* to nitrite and GSNO (Mukhopadhyay *et al.*, 2004)
4. The FNR regulon of *E. coli* and the effect of nitrate and nitrite on expression (Constantinidou *et al.*, 2006)
5. The transcriptional response of *E. coli* to nitric oxide under chemostat conditions (Pullan *et al.*, 2007)

applied in these experiments, NO is produced or present during the detoxification of all of these chemicals.

In addition to these three genes of known function, six genes of unknown function were also up-regulated in response to several types of nitrosative stress in two or more of the independent studies (table 1.1, group 2). These genes of unknown function include the 'prismane' or hybrid cluster protein, Hcp, and its reductase, Hcr, which have long been associated with nitrosative stress and has been designated as a hydroxylamine reductase, albeit in physiologically irrelevant conditions (Kim *et al.*, 2003; Wolfe *et al.*, 2002). The most significantly up-regulated gene of unknown function in three out of four of the micro-array studies was *ytfE*. The protein, YtfE, and its gonococcal homologue, DnrN, have been shown to repair nitrosylated iron-sulphur centers in both metabolic enzymes and transcription factors, which will be discussed in more detail in the following sections (Justino *et al.*, 2007; Overton *et al.*, 2008). The final two genes of unknown function, whose expression was up-regulated in two or more of the micro-array studies, were the two-gene operon, *yeaR-yoaG,* and the predicted gene, *ygbA*. One significant aspect of the regulation of these two genes was the fact that up-regulation was highest in the presence of nitrite, a known source of nitric oxide, when expression in a strain lacking functional FNR was compared to the expression in the isogenic parent strain (Constantinidou *et al.*, 2006). Several other genes were shown to be regulated in a similar way by this study but were not up-regulated in response to other sources of nitrosative stress (table 1, group 3). These include the operon of unknown function, *yibIH,* and the gene encoding the O^6-alkylguanine transferase DNA repair enzyme, *ogt* (Constantinidou *et al.*, 2006). This raises three interesting possibilities: first, that these genes are regulated in a novel, FNR-independent mechanism in response to nitrate and nitrite; secondly, that the gene products are involved in a stress response when nitrosative stress is so severe that FNR has been deactivated; and thirdly, that the regulatory response, and the different array of genes expressed in response to these different sources of nitrosative stress, may indicate the existence

of independent pathways for the detoxification of these very different reactive nitrogen species, and the different damage they cause in the cell including DNA repair.

The response of the bacterium, UPEC (Uropathogenic *Escherichia coli*), the primary cause of urinary tract infections, to acidified nitrite have been investigated by micro-array analysis. In response to acidified nitrite, a source of exogenous NO, a group of genes were up-regulated that included the genes of known function, *norV, hmpA, ytfE,*and *hcp-hcr* but more significantly, *yeaR-yoaG* (Bower *et al.*, 2009). The transcript encoding *yeaR* has also been shown to be one of the top 50 genes up-regulated during urinary tract infection in bacteria isolated from human volunteers (Snyder *et al.*, 2004). Exposure of UPEC bacteria to RNS enhances colonization of the host's urinary tract and can therefore be considered as important to the pathogenicity of this pathotype (Bower *et al.*, 2009).

For these reasons, the regulation and physiological function of *yeaR-yoaG* and *ogt*, as well as further investigation into the physiological function of HCP, will be investigated.

The role of YtfE in the repair of nitric oxide damaged iron-sulphur centres

The *ytfE* gene has been shown to be expressed in response to nitrosative stress, as discussed previously. Recent investigation has shown that *ytfE* expression is repressed by NsrR, the regulator of nitrosative stress, and is predicted to be activated by NarL in response to nitrate or nitrite (Constantinidou *et al.*, 2006; Filenko *et al.*, 2007). Physiological study of YtfE has shown that it fulfills an essential role in repair of proteins that contain iron-sulphur centres, including the citric acid cycle enzymes, aconitase B and fumarase A, as well as the transcription factor, FNR, in diverse bacterial species (Justino *et al.*, 2007; Overton *et al.*, 2008). How this protein functions and the mechanism by which it repairs Fe-NO adducts is not fully understood. It is possible that *ytfE* and other genes, such as *yeaR-yoaG,* that are regulated under similar environmental conditions could code for proteins involved in several parts of a single repair pathway for dealing with damaged iron-sulphur centers. Alternatively, they might

be involved in independent pathways, repair different damaged proteins or deal with the many side-products of NO chemistry discussed earlier.

Aims of this project

The aims of this project were to understand the regulatory response of *E. coli* to reactive nitrogen species and how the bacterium is able to deal with nitric oxide and the toxic secondary products generated from NO, and to repair the damage to cellular components that both NO and its derivatives can cause. Micro-array analysis has indicated that some of the genes implicated in nitrosative stress management may be regulated in a novel way, a necessity due to the effect of NO on essential transcription factors such as FNR. How these genes, in particular *yeaR-yoaG* and *ogt,* are regulated and the involvement of transcription factors such as FNR, NarL/P and NsrR at the *yeaR-yoaG* promoter was the main focus of this investigation.

As has been demonstrated, the chemistry of nitric oxide and the damage it causes is complex and may involve several pathways for repair and protection. The regulation of the genes *hcp-hcr* and *yeaR-yoaG*, as well as *ogt* and *yibIH*, by environmental conditions where nitric oxide would be abundant has implicated them as being involved with one or several pathways for nitric oxide detoxification. The physiological function of these genes, in particular *hcp-hcr* and *yeaR-yoaG,* and the mechanism by which they function was investigated during this project in an attempt to understand the ability of *E. coli* to persist and grow under conditions of nitrosative stress.

Chapter 2

Materials and methods

Materials

Suppliers

Unless otherwise stated, all chemicals and media were supplied by Sigma Aldrich or Oxoid. Oligodeoxynucleotides were supplied by Alta Biosciences at the University of Birmingham. Restriction endonucleases, calf intestinal alkaline phosophatase, T4 DNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs. PCR Supermix and glycogen were supplied by Invitrogen. Reverse transcriptase was supplied by Promega and RNAP holo-σ^{70} enzyme was purchased from Epicentre Biotechnologies. All radionucleotides were obtained from Perkin Elmer or MP Biomedical. DNase I enzyme was purchased from Roche and all pressurised gases were purchased from BOC Specialist Gases. All enzymes were used according to the manufacturer's instructions and in the buffers provided by the supplier. Purified Fis protein, which was purified by the method described previously (Osuna *et al.*, 1991), was kindly donated by Professor Rick Gourse (University of Wisconsin).

Media

Lennox broth (LB) contained 20 g l^{-1} tryptone, 10 g l^{-1} yeast extract and 10 g l^{-1} NaCl. Minimal salts medium (MS) contained 30 mM KH_2PO4, 60 mM K_2HPO_4, 7.5 mM $(NH_4)_2SO_4$, 200 μM $MgSO_4.7H_2O$, 2 mM trisodium citrate and was supplemented with 10 ml ammonium molybdate (1 mM), 10 ml sodium selenate (1 mM) and 10 ml of *E. coli* sulphur free salts per litre. The composition of *E. coli* sulphur free salts was: 0.3 M $MgSO_4 \cdot 7H_2O$; 50 mM $MnCl_2 \cdot 4H_2O$; 20 mM $FeCl_2 \cdot 6H_2O$ and 5 mM $CaCl_2 \cdot 6H_2O$, supplemented with 20 ml of concentrated HCl per litre of distilled water. All medium was dissolved fully, adjusted to pH 7.0 then sterilised by autoclaving at 121 °C and 15 psi for 15 min. Unless otherwise stated,

minimal medium was supplemented with 5% LB, 20 mM fumarate, 20 mM TMAO and 0.4% (v/v) glycerol as a carbon source.

Solid medium

Nutrient agar plates contained 28 g l^{-1} of nutrient agar (Oxoid) in distilled water. MacConkey lactose agar contained 40 g l^{-1} of MacConkey lactose agar dissolved in distilled water. LB agar contained 20 g l^{-1} tryptone, 10 g l^{-1} yeast extract, 10 g l^{-1} NaCl and 1.25% (m/v) bacteriological agar. All solid medium was autoclaved and cooled to 60 °C before the addition of antibiotics. Agar plates were prepared by pouring ~20 ml of cooled, molten agar into a 30 ml petri dish and allowed to solidify at room temperature. Plates were stored at 4 °C for 4 weeks and dried for 30 min. at 60 °C immediately prior to use.

Antibiotics

Stock antibiotics were added to solid and liquid medium to give the final working concentrations: 80 μg ml^{-1} ampicillin (Amp); 30 μg ml^{-1} chloramphenicol (Cm); 35 μg ml^{-1} tetracycline (Tet) and 50 μg ml^{-1} kanamycin (Kan). Ampicillin and kanamycin were prepared by dissolving powdered antibiotic in distilled water and filter sterilising. Solutions were stored at 4°C for 4 weeks. Chloramphenicol was dissolved in 100% ethanol, filter sterilised and stored at -20°C for 4 weeks and tetracycline was prepared by dissolving in a 50% (v/v) solution of ethanol in distilled water, filter sterilised and stored at -20°C for 4 weeks.

Buffers and solutions

Buffers for β-galactosidase activity assay

ONPG (*o*-nitrophenyl-β-D-galactopyranoside) was dissolved in 'A buffer' to give a working concentration of 13 mM. 'A' buffer consisted of 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 at pH 7. Assay buffer (Z-buffer) consisted of 10 mM KCl, 1 mM $MgSO_4.7H_2O$, 60 mM Na_2HPO_4, 30 mM $NaH_2PO_4.2H_2O$ supplemented with 2.7 ml β-mercaptoethanol per litre of distilled water, adjusted to pH 7.

Buffers for electrophoresis of DNA

Stock 5X TBE was prepared in distilled water and contained 0.445 M Tris (pH 8), 0.445 M boric acid and 10 mM EDTA. For agarose gel electrophoresis, a 5-fold dilution was made to give 1X TBE and for polyacrylamide DNA electrophoresis a 20-fold dilution was made to give 0.25X TBE. Agarose gels for DNA electrophoresis were prepared by dissolving 0.8% type II agarose (Sigma) in 1X TBE and boiling gently in a microwave. DNA blue loading sample dye consisted of 0.025% (w/v) bromo-phenol blue, 10% (v/v) glycerol, 10 mM tris-HCl at pH 7.5 and 1 mM EDTA in distilled water. For polyacrylamide gel electrophoresis of DNA, a stock solution of 7.5% acylamide was made consisting of 125 ml 30% (w/v) acrylamide, 0.8% bisacrylamide stock solution, 100 ml 5xTBE, 20 ml glycerol made up to 500 ml with distilled water. For a small gel 10 ml of the 7.5% acrylamide stock solution was mixed with 100 µl ammonium persulphate and 10 µl TEMED (N,N,N',N',-Tetramethylethylenediamine) and poured between two spaced glass plates.

Denaturing gel electrophoresis of proteins

Precast NuPAGE Novex 4-12% Bis-Tris gels were purchased from Invitrogen and used in a XCell SureLock™ Mini-Cell system as per manufacturer's instructions. SDS-PAGE loading buffer consisted of 2 g SDS, 20 ml glycerol, 5 mg bromphenol blue, made up to 92 ml using 0.1 x stacking gel buffer. Stacking gel buffer consisted of 1.25 M Tris, 1% (m/v) SDS and 5 ml l^{-1} TEMED adjusted to pH 6.8. Prior to use, 87 µl β-mercaptoethanol was added to a 1 ml aliquot of loading buffer stock.

Buffers for Coomassie staining of protein gels

Coomassie stain solution contained 50% (v/v) methanol, 10% (v/v) acetic acid and 2 g l^{-1} Coomassie brilliant blue. Fast de-stain solution contained 40% (v/v) methanol, 10% (v/v) acetic acid and shrink solution consisted of 48% (v/v) methanol, 2% (v/v) glycerol.

Buffers for silver staining of protein gels

All buffers for silver staining were supplied as part of the SilverQuest™ Silver Staining Kit supplied by Invitrogen. All buffers were used as per manufacturer's instructions.

Extraction and purification of nucleic acids

Phenol/ chloroform was purchased from Fischer Scientific and was composed of a 25:24:1 ratio of phenol, chloroform and isoamyl alcohol at pH 8.0. Ethanol was used at 100% and 70% by volume and kept ice-cold at all times.

Buffers and solutions for electromobility shift assay

FNR binding buffer, which was used for all electromobility shift assay binding reactions, consisted of 1 M potassium glutamate, 10 mM EDTA, 100 mM K_2HPO_4, 100 mM KH_2PO_4 at pH 7.5 and 500 μM DTT. Fix solution consisted of 10% (v/v) methanol and 10% acetic acid (v/v) in distilled water. DNA fragments were radiolabelled using [γ^{32}P]-ATP: 3000 Ci mmol^{-1}, 10 mCi ml^{-1} (Perkin Elmer). Unincorporated radionucleotide was removed using G50 Sephadex, consisting of 5 g Sephadex G-50 (Pharmacia Biotech) autoclaved in 100 ml TE buffer. Sephadex was washed three times in 150 ml TE buffer and finally suspended in 50 ml TE buffer and stored at 4 °C until needed.

Solutions for DNase footprints

DNA fragments were radiolabelled using [γ^{32}P]-ATP (7000 Ci mmol^{-1}, 100 mCi ml^{-1}) (MP Biomedical). Binding buffer for footprint reactions contained 20 mM Hepes pH 8.0, 5 mM $MgCl_2$, 50 mM potassium glutamate, 30 μg ml^{-1} herring sperm DNA, 0.5 mg ml^{-1} BSA and 1 mM DTT, dissolved in sterile water. DNase I dilution buffer contained 20 mM Hepes pH 8.0, 7.5 mM $MgCl_2$, 5 mM $CaCl_2$, 25 mM potassium glutamate and 62.5 mM KCl dissolved in sterile water. Stop solution contained 0.3 M sodium acetate and 10 mM EDTA. Gel loading buffer consisted of 40% deionised formamide, 5 M urea, 5 mM NaOH, 1 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanole dissolved in sterile water.

Buffers for potassium permanganate footprinting

DNA fragments were radiolabelled using [γ^{32}P]-ATP (7000 Ci mmol^{-1}, 100 mCi ml^{-1}) (MP Biomedicals). Binding buffer (10X) for footprint reactions contained 200 mM Hepes pH 8.0, 50 mM $MgCl_2$ and 500 mM potassium glutamate in a total volume of 20 µl per reaction. RNA polymerase was diluted to working concentration with transcription buffer containing 40 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl and 1 mM DTT. Potassium permanganate solution was prepared fresh for each at a concentration of 200 mM. Reaction stop solution contained 3 M ammonium acetate, 0.1 mM EDTA and 1.5 M β-mecaptoethanol. Gel loading buffer consisted of 95% v/v deionized formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanole FF.

Buffers for Western blotting

Transfer buffer contained 40 mM glycine, 50 mM Tris base and 5% (v/v) methanol, adjusted to pH 8.3. Tris buffered saline at ten times strength (10 x TBS) consisted of 200 mM Tris and 1.4 M NaCl, adjusted to pH 7.6. Blocking buffer contained 0.1 % (v/v) Tween-20 and 5% (m/v) non-fat dry milk in 1 x TBS. Wash buffer (TBS/T) contained 0.1% (v/v) Tween-20 in 1 x TBS.

Buffers for protein pull down and DNA-protein sampling experiments

Extraction buffer consisted of 50 mM Tris (pH 7.5), 50 mM NaCl, 10% (v/v) glycerol, 2 mM $MgCl_2$, 200 µg ml^{-1} phenylmethylsulfonyl fluoride (PMSF) and 4 µg ml^{-1} pepstatin. One complete Mini EDTA-free protease tablet (Roche) was added per 10 ml extraction buffer. This solution was supplemented with 500 µg ml^{-1}lysozyme, 0.2% (v/v) Tween-20 and 0.2% (v/v) Triton X100 after cells were resuspended. For DNA-sampling reactions 500 µg ml^{-1} RNAse was included, for protein pull downs 500 µg ml^{-1} RNase and 20 mg ml^{-1} DNase was included. Wash buffer contained 20 mM Hepes-Na (pH 7.4), 150 mM NaCl and 0.1% (v/v) Tween-20 in distilled water. Elution buffer consisted of 0.3 M NH_4OH and 0.5 M EDTA (pH 8). Gel

loading dye consisted of 10 mM *tris*(2-carboxymethyl)phosphine-HCl (TCEP-SB) (Sigma). After heating samples, 50 mM iodoacetemide was added to the sample mixture.

Buffers for de-staining, trypsinisation and FT-ICR mass spectrometry preparation

For Coomassie stained gels, de-stain solution consisted of 50% (v/v) methanol and 100 mM ammonium bicarbonate (ABC). For silver stained gels, de-stain solutions were supplied as part of the SilverQuest silver staining kit (Invitrogen) and used according to manufacturers instructions. Gel slice wash buffer contained 100 mM ammonium bicarbonate and 30% (v/v) acetonitrile in ultrapure water. Digestion buffer contained 50 mM ammonium bicarbonate and 5-10 ng μl^{-1} porcine trypsin (Promega). Peptide extraction buffer contained 50% (v/v) acetonitrile and 0.1% triflouroacetic acid (TFA).

Solutions for NADH-dependent nitrite reductase activity assay

All buffers for the preparation of cell extract containing NirB and the assay of NirB activity contained 50 mM Tris HCl and 5 mM EDTA at pH 8 (TE). This buffer was supplemented with 5 mM ascorbate (TEA); 5 m M ascorbate and 1 mM NO_2^- (TEA-NO_2) ; 5 mM ascorbate and 10 μM FAD (TEA-FAD) or 5 mM ascorbate, 1 mM NO_2^- and 10 μM FAD (TEA-NO_2^- FAD) dependent upon the requirements at each stage.

Preparation of nitric oxide saturated water

Pure nitric oxide gas was used to saturate a sealed vial of sterile distilled water, adjusted to pH 3 using HCl. The vial was first sparged with nitrogen to remove dissolved oxygen from the water. Nitric oxide gas was scrubbed first through a 2 M solution of sodium hydroxide to remove impurities and secondly through distilled water to remove aerosolised sodium hydroxide before entering the saturation vial. After exiting the saturation vial, excess nitric oxide was scrubbed through a 2 M solution of potassium permanganate to limit release into the atmosphere. The concentration of stock nitric oxide saturated water (NOSW) was 2 mM.

Bacterial methods

Strains

Bacterial strains used in this study are listed in table 2.1. Bacteria were purified by streaking to single colony on nutrient agar plates supplemented with appropriate antibiotics. Plates were incubated overnight at 37°C unless otherwise stated. Bacteria on plates were stored at 4°C for up to six weeks. For longer storage, 0.75 ml of an overnight liquid culture was mixed with 0.25 ml of a sterile 60% (v/v) solution of glycerol and stored at -70°C.

Plasmids

Plasmids used in this study are listed in table 2.2 and represented diagrammatically in figure. 2.1 to 2.9

Growth of bacteria

A single colony from a freshly streaked plate was used to inoculate 1 to 5 ml of LB, supplemented with appropriate antibiotics, and aerated vigorously at 37°C overnight.

Growth experiments in the presence of nitrosative stress

For measurement of the effect of nitrosative stress, 100 ml of minimal medium supplemented with 5% LB, 0.4% glycerol, 20 mM fumarate and 20 mM TMAO in a 100 ml conical flask was inoculated with 1% of an overnight culture. The culture was incubated at 37°C without shaking until an optical density of 0.3 was reached, at which point the culture was stressed with either nitric oxide saturated water or hydroxylamine. The effect of nitrosative stress was monitored by the effect on growth compared to an isogenic, untreated culture.

β-galactosidase activity assay

The substrate for the measurement of β-galactosidase activity was the colourless lactose analogue, ortho-nitrophenyl-β-galactopyranoside (ONPG), which is converted to the yellow product o-nitrophenol by the enzyme β-galactosidase. The bacterial strains used for the

Table 2.1 All *E. coli* K-12 stains used in this study

Strain	Description	Reference or source
DJS100	Derivative of MG1656 engineered to express a 3xFLAG tagged NarL protein	This study
DJS101	JCB387Δ*narL-flag*::Kan^R	Transduce JCB387 to Kan^R with P1 phage propagated on DJS100
DJS102	*Δfnr::Cm^R ΔnarL*-FLAG::Kan^R derivative of JCB387	Transduce JCB3911 to Kan^R with P1 phage propagated on DJS100
JCB1001	Δ*fnr*::Cm^R derivative of MG1655	(Browning *et al.*, 2000)
JCB387	*E. coli* RV *Δnir* Δ*lac*	(Page *et al.*, 1990)
JCB3871	Δ*fis::*Str^R derivative of JCB387	(Wu *et al.*, 2004)
JCB38712	Δ*fnr*::Cm^R derivative of JCB3871	Transduce JCB3901 to Cm^R with phage P1 propagated on JCB3911
JCB3875	Δ*narP*252::Tn*10*d(Cm) derivative of JCB387	(Page *et al.*, 1990)
JCB3883	Δ*narL* derivative of JCB387	(Tyson *et al.*, 1994)
JCB3884	*narL narP*252::Tn*10*(Cm) derivative of JCB387	(Tyson *et al.*, 1994)
JCB3886	Δ*narX*::Kan^R derivative of JCB387	Transduce JCB387 to Kan^R with phage propagated on JWK1213-1
JCB38860	Δ*fnr^S* Δ*narX*::Kan^R derivative of JCB387	Transduce Δ*fnr^s* to Kan^R with phage propagated on JWK1213-1
JCB3887	Δ*narQ*::Cm^R derivative of JCB387	Transduce JCB387 to Cm^R with phage propagated on RK4353Δ*narZ*::Str^R Δ*narQ*::Cm^R
JCB38870	Δ*fnr^S* Δ*narQ*::Cm^R derivative of JCB387	Transduce Δ*fnr^s* to Cm^R with phage propagated on RK4353Δ*narZ*::Str^R Δ*narQ*::Cm^R
JCB3888	Δ*narX*::Kan^R Δ*narQ*::Cm^R derivative of JCB387	Transduce JCB387 Δ*narX*::Kan^R to Cm^R with phage propagated on JWK1213-1
JCB38880	Δ*fnr^S* Δ*narX*::Kan^R Δ*narQ*::Cm^R derivative of JCB387	Transduce Δ*fnr* Δ*narX::kan^R* to Cm^R with phage propagated on RK4353Δ*narZ*::Str^R Δ*narQ*::Cm^R
JCB3901	Δ*nsrR*::Km^R derivative of JCB387	Transduce JCB387 to Kan^R with phage P1 propagated on JOEY 60
JCB3902	Δ*fnr*::Cm^R derivative of JCB3901	Transduce JCB3901 to Cm^R with phage P1 propagated on JCB3911
JCB3911	Δ*fnr*::Cm^R derivative of JCB387	Transduce JCB387 to Cm^R with phage P1 propagated on JCB1001

JCB39110	Δ*fnr*^{S} derivative of JCB387	'Cured' chloramphenicol resistance of JCB3911 with pCP20
JCB3983	Δ*nsrR*::Kan^{R} derivative of JCB3883	Transduce JCB3883 to Kan^{R} with phage P1 propagated on JOEY 60
JCB3984	Δ*nsrR*::Kan^{R} derivative of JCB3884	Transduce JCB3884 to Kan^{R} with phage P1 propagated on JOEY 60
JCB4983	Δ*nsrR*::Kan^{R} Δ*fnr*::Cm^{R} derivative of JCB3883	Transduce JCB3983 to Cm^{R} with phage propagated on JCB3911
JCB4984	Δ*nsrR*::Kan^{R} Δ*fnr*::Cm^{R} derivative of JCB3884	Transduce JCB3984 to Cm^{R} with phage propagated on JCB3911
JCB4999	RK4353Δ*hcp*::cat	(Filenko, N. PhD Thesis 2005 University of Birmingham)
JCB5000	RK4353Δ*hcp*	(Filenko, N. PhD Thesis 2005 University of Birmingham)
JCB5100	RK4353Δ*yeaR-yoaG*::cat	Constructed by Lesley Griffiths for this study
JM109	*lacZ recA*	Promega
JOEY 60	*araD139* (*ara-leu*) (*codB-lac*I) *galK16 galE15 relA1 rpsL spoT1 nsrR*::Kan^{R}	(Bodenmiller and Spiro, 2006)
JWK1213-1	Δ*narX*::Kan^{R} derivative of MG1655	H. Aiba
MG1655*lacI*-3*x*FLAG	derivative of MG1655 engineered to express a 3xFLAG tagged version of LacI	Dave Lee (University of Birmingham) unpublished data
RK4353	*E. coli* K-12 *ΔlacU169 ara139 rpsL gyrA*	(Stewart and MacGregor, 1982)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17*	Stratagene

Table 2.2 All plasmids and promoter fragments used in this study

Name	*Description*	*Source*
Plasmids		
pACBSR-DL1	Derivative of pACBSR encoding the yeast meganuclease, *Sce*I and the λ Red recombinase system (*gam* only) under the control of the *ara* promoter. Confers Cm^R (fig 2. 4).	(Butala *et al.*, 2009)
pCP20	Confers Amp^R and Cm^R resitance and a temperature sensitive origin of replication. Expresses the FLP recombinase.	(Cherepanov and Wackernagel, 1995)
pDJS901	The vector pRW902 carrying the *yeaR100* promoter fragment for use in promoter DNA sampling experiments (fig 2.8)	This study
pDOC-F	Encodes a 3 X FLAG tag and kanamycin resistance for use as a template for Datsenko and Wanner mutagenesis. Also contains Flp sites necessary for antibiotic 'curing' (fig 2.6).	(Lee *et al.,* in submission)
pGEM-Teasy©	The vector was used for direct cloning of PCR products and blue/ white screening of recombinants. (fig 2.3)	Promega
pKD46	Encodes the bacteriophage lambda red recombinase system under the control of the arabinose inducible *araBAD* promoter. Encodes ampicillin resistance (*bla*). (fig 2.9)	(Datsenko and Wanner, 2000)
pRW50	Broad-host-range *lacZ* fusion vector for cloning promoters as *Eco*RI-*Hin*dIII fragments: contains the RK2 origin of replication and encodes Tet^R (see fig.2.1). Used for all β-galactosidase assays in this study unless otherwise stated (fig 2.1)	(Lodge *et al.*, 1992)
pRW902	Derivative of pRW50 modified in the linker region to contain 5-LacI operator sequences immediately upstream of an *Eco*RI- *Hin*dIII cloning site, flanked by *Sce*I sites. Encodes Tet^R (fig 2.5)	(Butala *et al.*, 2009)
pSR	High copy number pBR322 derivative, which carries the Lambda *oop* terminator. Used for cloning *Eco*RI-*Hin*dIII promoter fragments for use in *in vitro* transcription reactions (ampicillin resistant) (fig. 2.2)	(Kolb *et al.*, 1995)
pYEAR100	The vector pRW50 carrying the *yeaR100* promoter fragment cloned as a transcriptional fusion (fig 2.7)	This study

Promoter Fragments	**(All *Eco*R1-*Hin*dIII fragments)**	
ogt100	*E. coli ogt* promoter fragment carrying nucleotide sequences from -269 to +51	(Squire *et al.*, 2009)
ogt101	Fragment *ogt100* carrying C to T, T to A, C to G and T to G mutations at positions -80, -77, 75 and -73	Meng Xu (PhD Thesis)
ogt102	Fragment *ogt100* carrying C to G and T to C mutations at positions -84 and -73	(Squire *et al.*, 2009)
ogt104	Fragment *ogt100* carrying T to G and G to C mutations at positions -51 and -40	(Squire *et al.*, 2009)
yeaR100	*E. coli yeaR* promoter fragment carrying nucleotide sequences from -294 to +96	This study
yeaR102	Fragment *yeaR100* carrying A to C; T to A; C to T and A to T mutations at positions -20, -19, -18 and -17, respectively.	This study
yeaR200	Fragment *yeaR100* carrying T to A; G to T; C to T and T to C mutations at positions -81, -80, -79 and -78, respectively.	This study

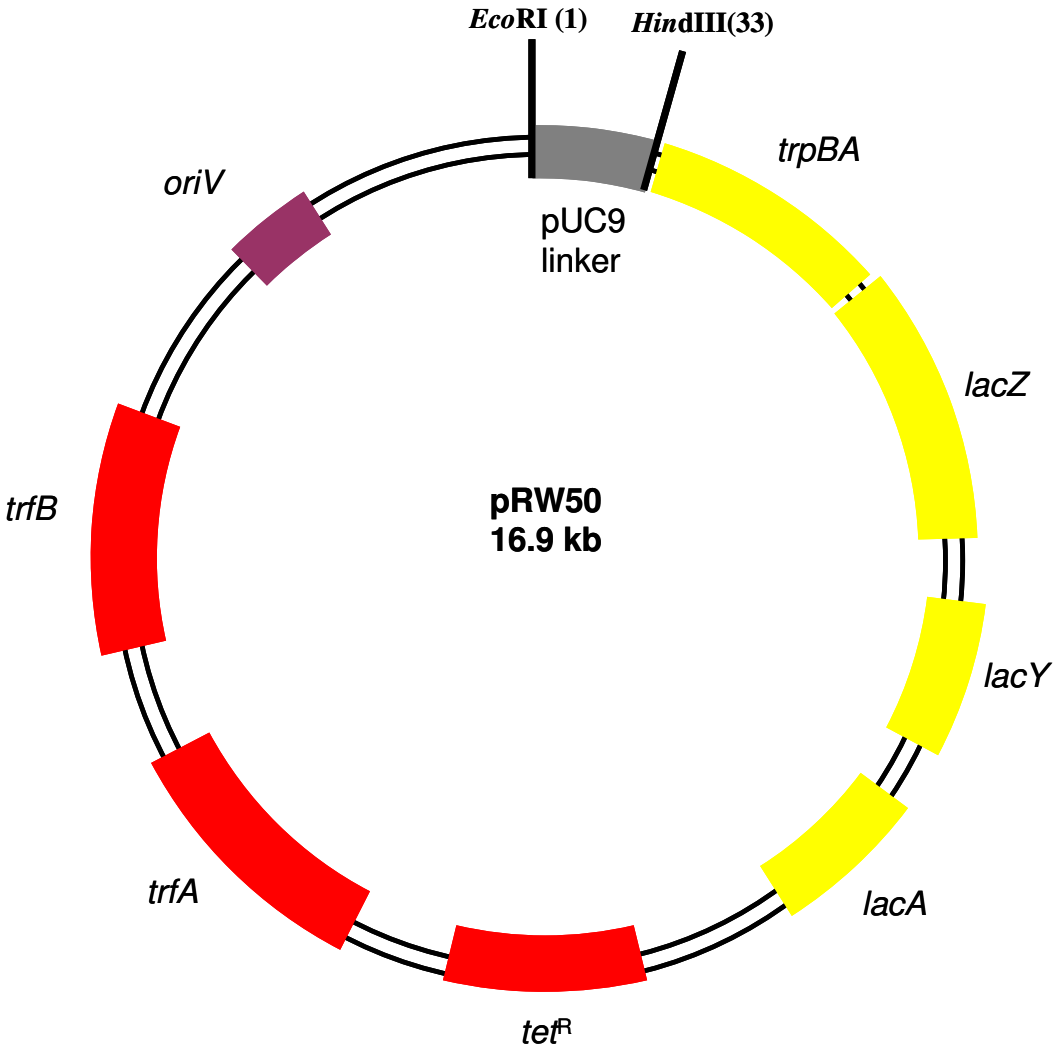

Figure 2.1 Diagram of the *lacZ* fusion vector, pRW50

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

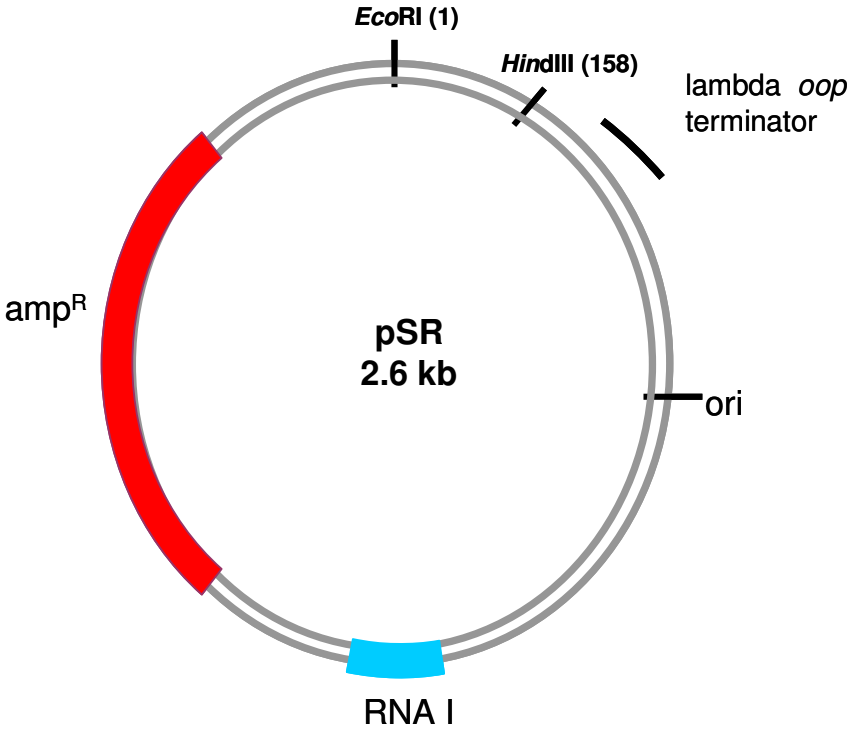

Figure 2.2 The multi-copy cloning vector, pSR

The figure shows a map of pSR-TB10, which carries an *Eco*RI-*Hin*dIII *melR* promoter fragment. *Eco*RI-*Hin*dIII promoter fragments are cloned upstream of the lambda *oop* terminator. pSR can be used in *in vitro* transcription reactions, in which transcription initiates at the cloned promoter and terminates at the lambda terminator, to produce a discrete transcript of a defined length. Also shown are the RNAI gene, which produces a control transcript during *in vitro* transcription, the ampicillin resistance marker (*amp*R) and the origin of replication (*ori*).

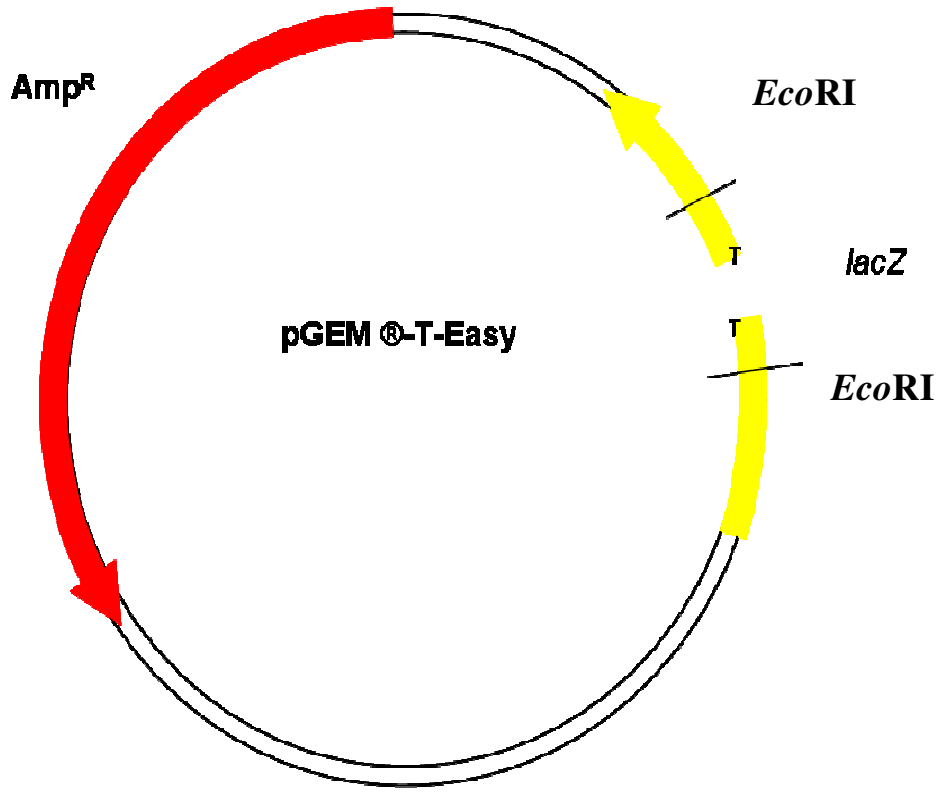

Figure 2.3 The high-copy number cloning vector, T-easy©

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

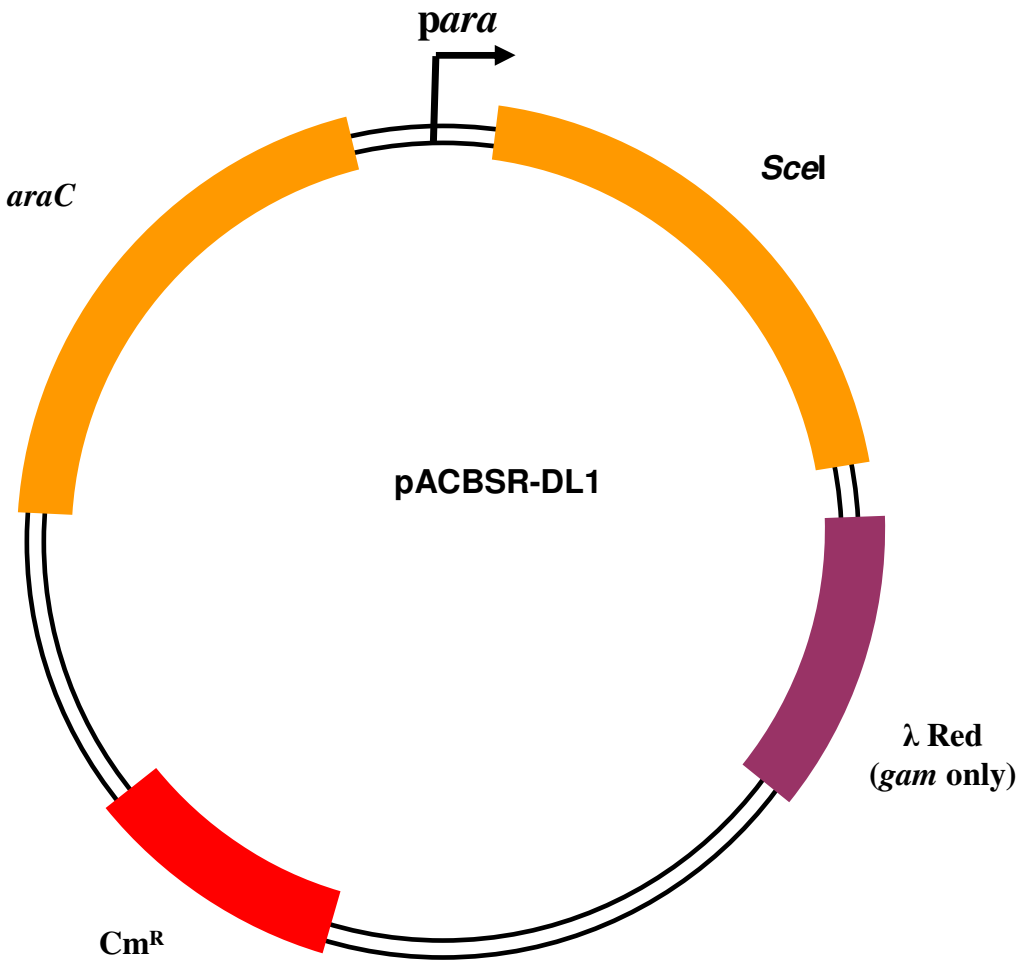

Figure 2.4 The plasmid, pACBSR-DL1

A schematic representation of the DNA-sampling plasmid pACBSR-DL1, a derivative of pACBSR available from Scarab Genomics (Butala *et al*. 2009). This plasmid encodes the yeast meganuclease I-*Sce*I and the λ-Red recombinase gene, *gam*, under the control of an arabinose inducible promoter, p*ara*. The plasmid also encodes resistance to chloramphenicol. The *araC* gene encodes an activator of p*ara.*

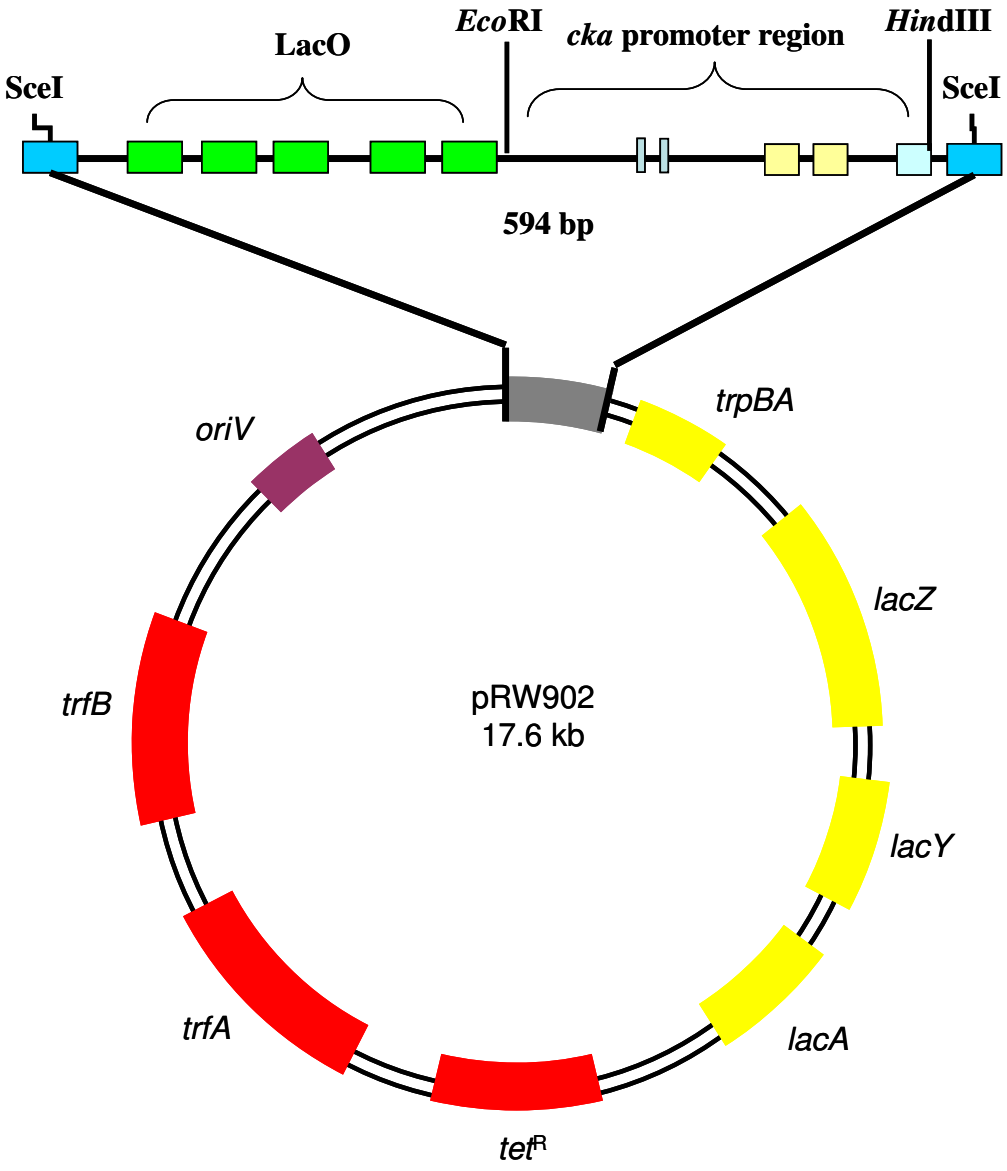

Figure 2.5 The DNA-sampling plasmid pRW902

A schematic representation of the plasmid pRW902, a derivative of pRW50 carrying a modified cloning region. The construct carries five LacI operator sites flanked on either side by a upstream of the *cka* promoter region, which is flanked by two target sites for the yeast meganuclease I-*Sce*I. Promoters regions to be sampled are cloned into the *Eco*RI and *Hin*dIII restriction sites, replacing the *cka* promoter region.

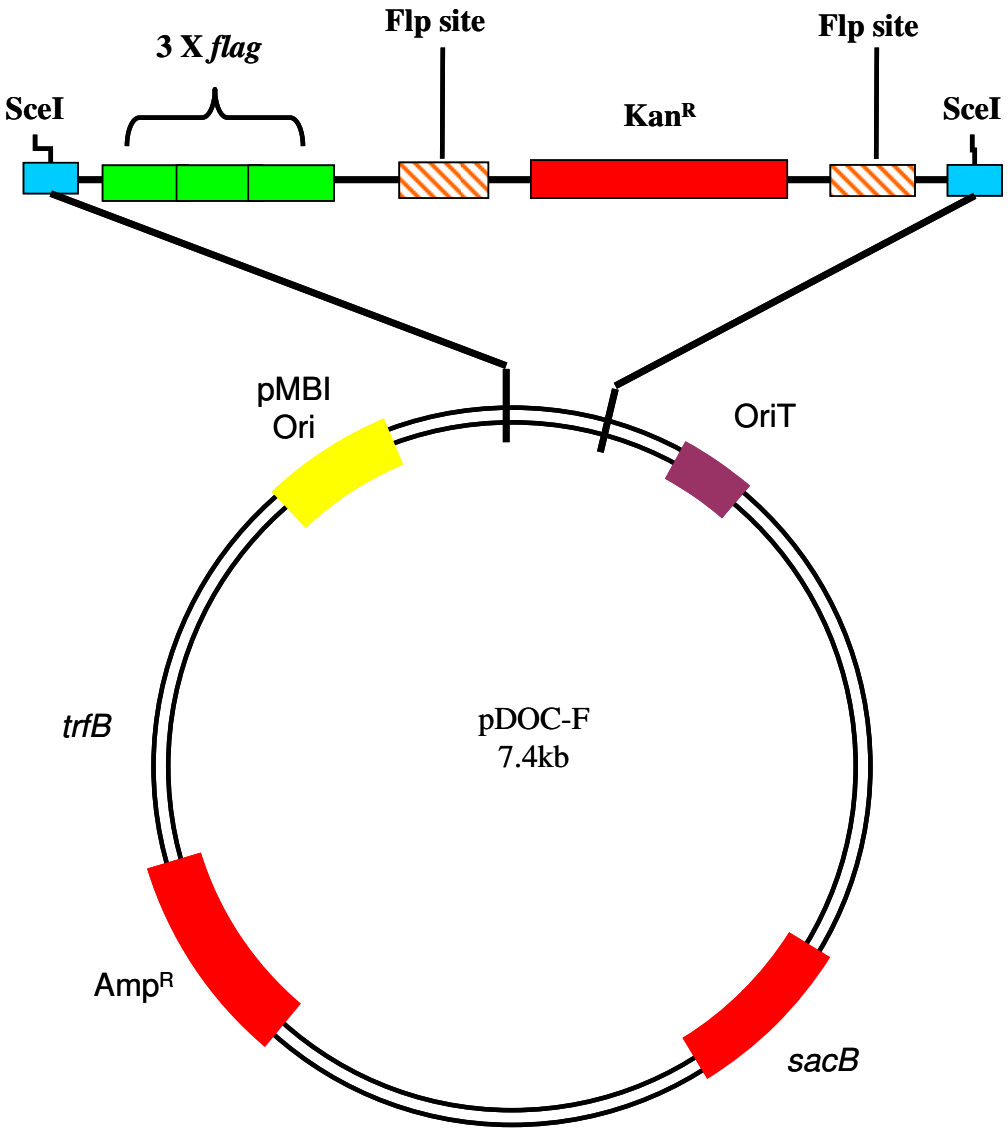

Figure 2.6 The template plasmid, pDOC-F, used for creating Flag-tag chromosomal mutations using the Datsenko and Wanner method

A schematic representation of the plasmid pDOC-F, encoding a 3 X Flag tag and a kanamycin resistance cassette. FLP recombinase sites are included either side of the Kan cassette to allow for 'curing' of the antibiotic resistance. The plasmid is used as a template for PCR generation of linear fragments. The flanking *Sce*I meganuclease sites facilitate 'gene gorging' if Datsenko and Wanner mutagenesis is unsuccessful.

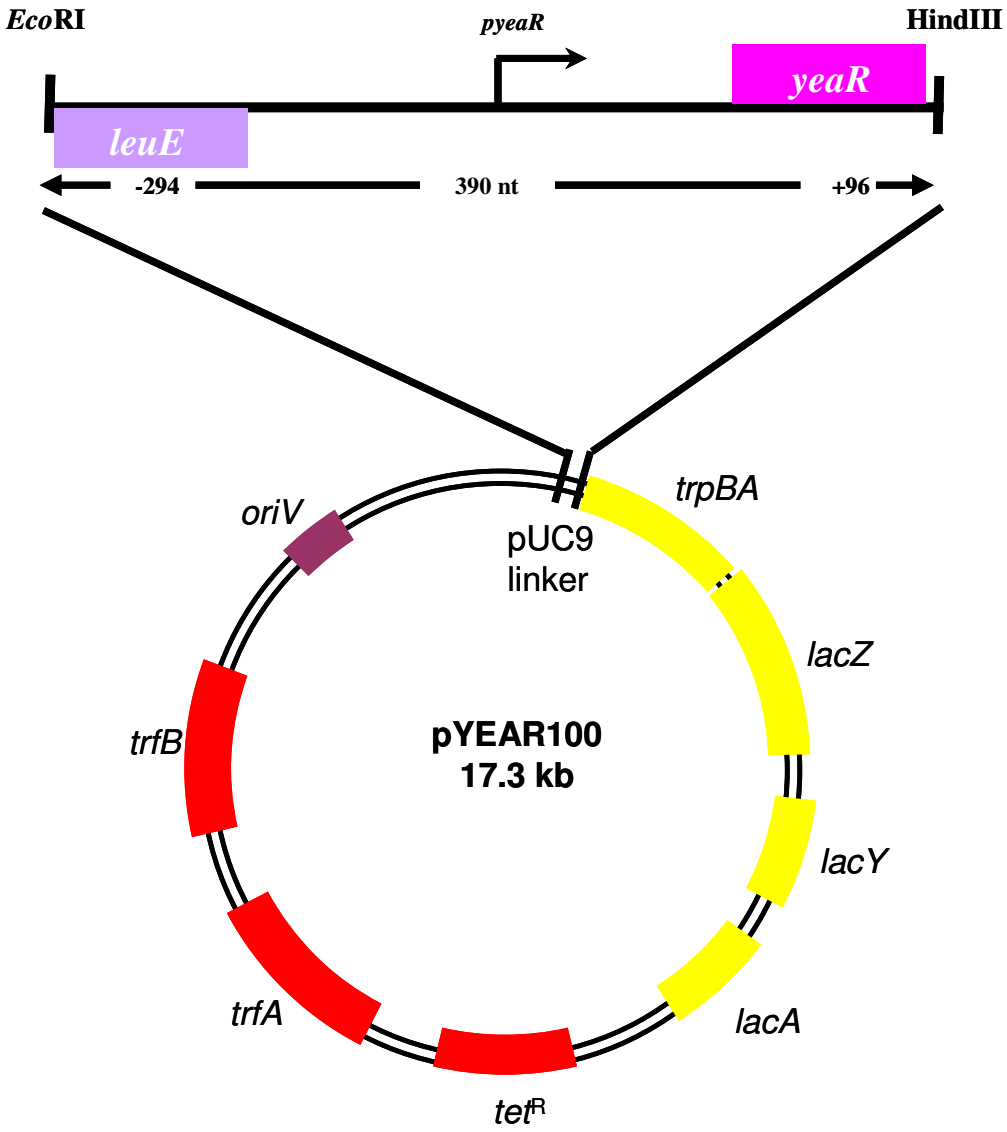

Figure 2.7 The *yeaR* promoter-*lacZ* fusion plasmid, pYEAR100

A schematic diagram of the *lacZ*-promoter fusion pYEAR100, constructed by cloning the *yeaR100* promoter fragment into pRW50 as an *Eco*RI/ *Hin*dIII fragment at the multiple cloning site. The *yeaR* promoter fragment includes sequence from -294 to +96 with respect to the *yeaR* transcription start site and includes coding DNA from *yeaR* and the upstream gene *leuE* flanked by introduced *Eco*RI and *Hin*dIII sites. This construct is used throughout this study for measuring *yeaR* promoter-driven expression of *lacZ*.

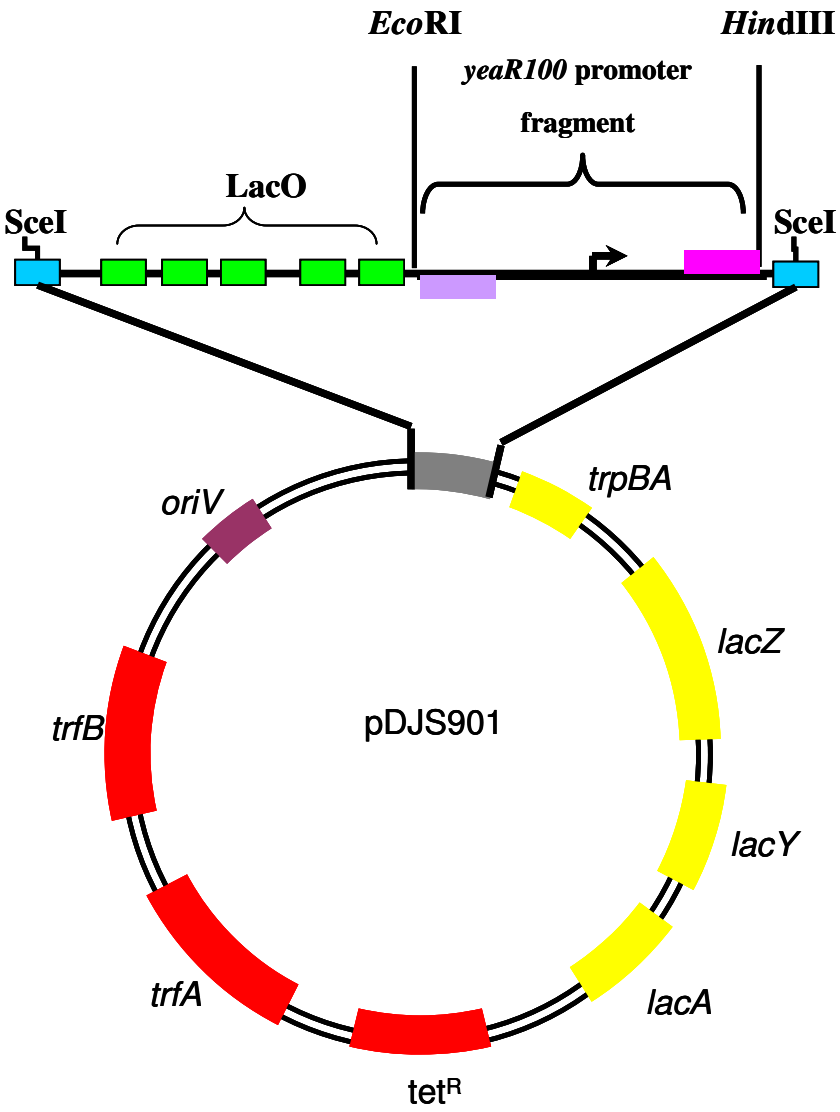

Figure 2.8 The *yeaR* promoter DNA-sampling plasmid, pDJS901

A schematic diagram of the *yeaR* promoter DNA-sampling plasmid, pDJS901, constructed by cloning the *yeaR100* promoter fragment into pRW902 as an *Eco*RI/ *Hin*dIII fragment at the multiple cloning site. This places the *yeaR100* promoter fragment immediately downstream of 5 LacI operator and flaked either side by an *Sce*I meganuclease site. This construct can be used for identifying unknown promoter-bound proteins using the DNA-sampling technique.

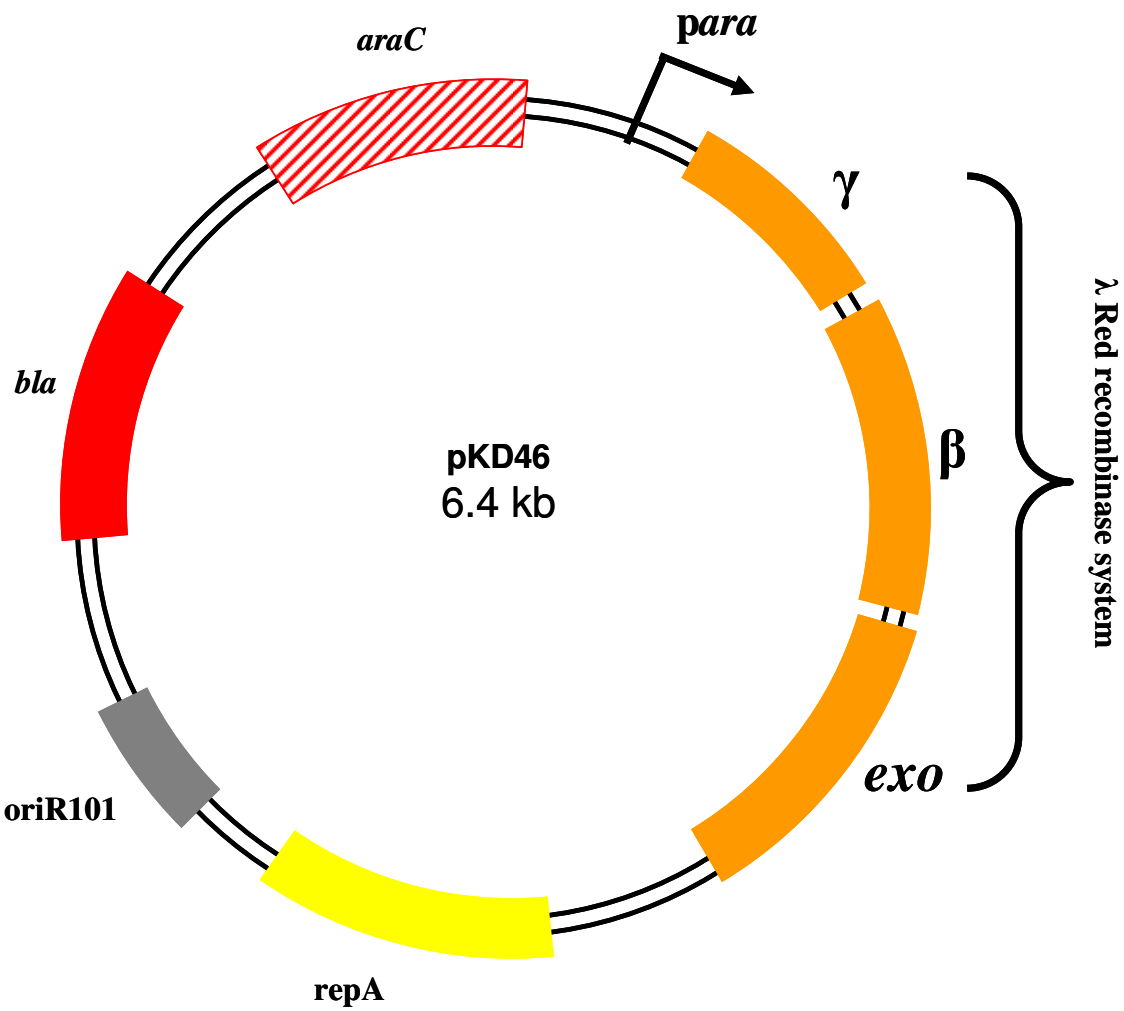

Figure 2.9 The λ Red recombinase encoding plasmid, pKD46

A schematic diagram of the plasmid, pKD46, which encodes the bacteriophage Lambda red recombinase system under the control of the arabinose inducible *araB*AD promoter. The plasmid encodes ampicillin resistance (*bla*) and contains the oriR101 origin of replication. The plasmid is used to recombine linear fragments of DNA into the *E. coli* chromosome.

determination of β-galactosidase activity were grown overnight in LB supplemented with the appropriate antibiotics as described previously. For aerobic growth, 50 µl of the overnight culture was used to inoculate 10 ml of fresh sterile LB in a 50 ml conical flask. Cultures were aerated vigorously until an optical density of 0.19 to 0.3 had been reached. For anaerobic growth experiments, the overnight culture was diluted 20-fold in either 10 ml of sterile medium in a test tube or 100 ml of medium in a 100 ml conical flask. For all anaerobic growth assays, the medium consisted of MS (minimal salts medium) supplemented with 5% LB, 0.4% glycerol, 20 mM fumarate and 20 mM trimethylamine N-oxide (TMAO) unless otherwise stated (Constantinidou *et al.*, 2006). If nitrate or nitrite dependent activity was to be measured, the medium was supplemented with either 20 mM nitrate or 2.5 mM nitrite. Anaerobic cultures were grown until the desired optical density was reached, at which point 2 ml of culture was removed to a sterile test tube and lysed using 20 µl of 1% (w/v) sodium deoxycholate and 20 µl toluene. The lysates were then incubated at 37°C for 30 min. to evaporate the toluene. Between 100 µl and 500 µl of each lysate was added to 2 fresh tubes per sample, containing the appropriate amount of Z-buffer to make a total volume 2 ml. The same amount of lysate was added to a third tube per sample, with the appropriate volume of Z-buffer to make the overall volume 2.5 ml, this tube was used as a control. The assay tubes were pre-warmed to 37°C before the reaction was initiated. The assay was started with the addition of 500 µl of pre-warmed ONPG (13 mM) to the two assay tubes. The samples were mixed by vortexing and the time taken for a yellow colour to develop was recorded. Once sufficient yellow colour had developed the reaction was stopped with the addition of 1 ml of 1 M sodium carbonate, which raises the pH over pH 9 and deactivated the β-galactosidase enzyme. The OD_{420} of the control tube containing no ONPG was subtracted from the average OD_{420} of the two sample tubes. The activity of each lysate was then calculated using the following equation:

$$\beta\text{-galactosidase activity} = \frac{1000 x OD_{420} x 2.5 x 3.5 x D}{t x 4.5 x V . OD_{650}}$$

Key:

2.5 = the conversion factor to convert the OD_{650} of the bacterial culture to mg dry cell mass on the assumption that an OD_{650} of 1 corresponds to 0.4 mg dry cell mass. ml^{-1}

3.5 = Total assay volume

4.5 = the molar extinction coefficient of ONP at 420nm

D = Dilution factor if lsyate was diluted (for high activity lysates)

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

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

t = time taken for yellow colour to develop (min)

V = volume of lysate used (ml)

Units are expressed as: nmol ONPG hydrolysed min^{-1} (mg dry cell mass) $^{-1}$

Large scale growth of bacteria

For preparation of cytoplasmic extracts for the measurement of NirB dependent NADH oxidation, a large volume of cells was needed. A 5 ml overnight culture was transferred to 100 ml of LB in a 500 ml conical flask per strain, which was aerated at 37°C for 8 hours. 20 ml from each culture were used to inoculate 3 flasks per strain containing 2 l of LB supplemented with 0.4 % glucose (w/v) and 20 mM nitrate. These flasks were then grown at 37°C without shaking until an OD_{650} of 0.3 was obtained. At this point 2 flasks were treated with a specific concentration of hydroxylamine and the third was harvested by centrifugation at 1000 g for 5 min. The pellet was mixed to slurry in an equal volume of TEA buffer and stored on ice. After 2 hours a second flask per strain was harvested in the same way and the third flask per strain at 5 hours post-stress. Once all pellets were harvested, resuspended and washed in TEA, bacteria

were again pelleted at 1000g for 5 min. before being mixed to slurry with an equal volume of TEA buffer.

Preparation of cell extracts

The bacterial slurry was broken in a French press pressure cell cooled to 4°C at a pressure of 60 Mpa (3.8 ton in^{-2}). Cytoplasmic extract was separated by high speed centrifugation at 75000g for 2 hours in an 8 x 50 ml rotor of an MSE 'superspeed 50' centrifuge and decanted into clean test tubes. All fractions were kept at 4°C.

Soluble protein quantification using the folin assay

The soluble protein concentration was determined as described previously (Lowry *et al.*, 1951). Under high pH conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a mono-valent form. Monovalent copper ions and the radical groups of tyrosine, tryptophan and cysteine react with Folin reagent to produce an unstable product that becomes reduced to tungsten blue. Standards were 20, 40, 60, 80 and 100 µg of bovine serum albumin (BSA) solution. Cytoplasmic extracts were diluted by 10 and 100-fold and 50 and 100 µl of each of the dilutions were used to determine the total protein concentration. Samples were diluted to 0.6 ml with distilled water and assayed in duplicate, A 3 ml sample of Folin B solution was added to each tube and the samples were mixed by vortexing. Samples were incubated at room temperature for 10 min. before 0.3 ml of Folin reagent was added to all tubes. Samples were well mixed and incubated for a further 30 min. at room temperature after which time the absorbance at 750 nm was determined. For the standards, a line of best fit was determined from the absorbance at 740 nM and the equation $y= ax+ b$ was used to calculate the amount of protein where: y= the amount of protein, x= the absorbance at 750 nm and a and b are the regression co-efficient.

NirB-dependent nitrite reduction assay

Nitrite reductase activities were assayed using the method described previously (Coleman *et al.*, 1978). The reaction was initiated by pipetting 50 µl of cytoplasmic extract directly into a 1 ml cuvette containing 950 µl of TEA buffer supplemented with 0.25 mM NADH, 1 mM NAD^+ and 2 mM $NaNO_2$. The decrease in absorption at 340 nm was observed over time using a spectrophotometer and a calibrated chart recorder. The rate of NADH oxidation in the absence of nitrite was also calculated and subtracted from the assay rate to give the NirB dependent NADH oxidation rate. The rate of NADH oxidation was calculated using the following equation:

$$\text{NirB activity} = \frac{\Delta A \times V \times 10^3}{\varepsilon \times E \times \psi}$$

Where:

ΔA= rate of change of NADH absorption per min.

V= volume of reaction in ml

ε= Molar extinction coefficient of NADH

E= volume of enzyme added

Ψ= protein concentration of cytoplasmic extract (mg ml^{-1})

Units are expressed as nmol NADH oxidised min^{-1} mg cytoplasmic protein $extract^{-1}$

Creation of MG1655*narL::*3xFLAG tagged strain using Datsenko and Wanner chromosomal mutagenesis

A chromosomal C-terminal *narL::*3xFLAG tagged mutant with a selectable kanamycin resistance cassette was constructed using a modified version of the Datsenko and Wanner (Datsenko and Wanner, 2000). The plasmid pDOC-F (fig 2.6) was used as a template to create a linear DNA fragment by PCR, using oligonucleotides DNarLFK fwd and DNarLFK rwd, listed in table 2.3. The upstream primer, DNarLFK, encoded the final 15 codons of *narL* excluding the stop codon followed immediately by the first codon of the 3xFLAG tag. The

Table 2.3 All oligonucleotide primers used in this study

Primer	***Sequence (5'→3')***	***Purpose***
D10520	CCCTGCGGTGCCCCTCAAG	Anneals upstream of the *Eco*RI site in pRW50. Used for sequencing and amplification of promoter inserts in this vector
D36245	GGGTTATTGTCTCATGAGC G	Anneals upstream of the *Eco*RI site of pSR vector. Used for sequencing of inserts in this vector and dideoxy sequencing calibration
D49724	GGTTGGACGCCCGGCATAG TTTTTCAGCAGGTCGTTG	Anneals 83 bp downstream of the *Hin*dIII site of pRW50. Used for synthesis of cDNA for use in primer extension assay
D66220	CTGGATATCACCGAAAGCA C	Anneals to *E. coli* chromosome upstream of *narL*. Used to confirm introduction of 3x*flag* tag and kanamycin cassette onto chromosome.
D66221	CAATCCCGTCGCGTTGAGT C	Complementary check primer to D66220, anneals downstream of *narL.*
DNarLFK fwd	GAATTCGTGGAAGCAGCGG TATGGGTGCATCAGCAGCG CATTTTCGACTACAAAGAC CATGACGGTG	Complementary to final 15 codons of *narL* excluding stop codon and 22 bp of pDOC-F vector sequence. Used to create NarL-3xFLAG tagged Kan^R derivative of MG1655
DNarLK rwd	GCTAGCCGATGCATTGTCA AACGACGAACTGCGCTGGG AACCGTAAATGAATATCCT CCTTAGTTC	Complementary to 45 bp of *E. coli* chromosome, immediately downstream of *narL* and 21 bp of pDOC-F vector. Reverse primer paired with DNarLFK fwd used to create NarL-3xFLAG tagged Kan^R derivative of MG1655
DSpyeaR for	ACCTGTGAATTCGCGACGC TGGAACTGGTG	Primer pair designed to amplify the promoter region of *yeaR* and engineer *EcoR1/ HindIII* sites into 5' and 3' ends, respectively, for sub-cloning
DSpyeaR rev	GTGAACAAGCTTCAGAAAG GCGTTGAGCGCG	
DYEAR102 for	CTAAAAAGTAACCAATAAA TGGTATTTAAAATGCAAAT TCATTGGCGTACCCTGAAA CGGCTG	Mutagenic primer designed to introduce mutations into the downstream half site of FNR 1 in the *yeaR-yoaG* promoter
DYEAR102 rev	CAGCCGTTTCAGGGTACGC CAATGAATTTGCATTTTAA	Complementary mutagenic primer to DYEAR102 for

	ATACCATTTATTGGTTACTTTTTAG	
DYEAR200 for	TCCTCCCTGATTCTTCGCTGATATGGATCCAAAAAGTAACCAATAAATGGTATTT	Mutagenic primer designed to introduce a *Bam*HI site at the position of FNR 2 in the *yeaR-yoaG* promoter
DYEAR200 rev	TAAATACCATTTATTGGTTACTTTTTGGATCCATATCAGCGAAGAATCAGGGAGGA	Complementary primer to DYEAR200for
T-easy FWD	GACGTCGCATGCTCCCGC	Primer designed to anneal upstream of the blunt-end cloning site of T-Easy for sequencing of ligated fragments.

downstream primer, DNarLFK, encoded 45 bp of the *E. coli* chromosome immediately downstream of the NarL stop codon and 21 bp of sequence homologous to the region of pDOC-F, downstream of the kanamycin resistance cassette. These plasmids were used with the pDOC-F plasmid to generate a linear fragment consisting of a region of DNA homologous to *narL* tagged to a 3xFLAG tag encoding region, followed by a kanamycin cassette and finally by a second region of DNA homologous to the sequence downstream of *narL.* This linear fragment was purified using a Qiagen PCR clean up kit, digested with *Dpn*I overnight to remove methylated template DNA, checked for purity and quantified by electrophoresis on a 0.8% agarose gel.

An overnight culture of strain MG1655, transformed with pKD46, was used to inoculate two 10 ml aliquots of sterile LB in 50 ml conical flasks. These cultures were shaken vigorously at 30°C until an OD_{650} of 0.3 was reached. At this point the culture was split between 4 sterile test tubes. Three of the cultures were treated with 0.4% L-arabinose, the fourth was left un-induced as a control. Tubes were incubated at 37°C with vigorous shaking for 1 hour and cells were harvested by centrifugation at 13, 000 r.p.m for 1 min. The supernatant was discarded and pellets were resuspended in 1 ml of 10% (v/v) ice-cold sterile glycerol. This wash was repeated three times. After the final centrifugation, the supernatant was discarded and cells were resuspended in the residual glycerol (~30 µl). Approximately 200 ng of the purified linear DNA fragment was added to three out of the four tubes, as described bellow, mixed gently and transferred to an electroporation cuvette.

Tube	***Arabinose***	**DNA**
1	+	200 ng of PCR product
2	+	200 ng of PCR product
3	+	-
4	-	200 ng of PCR product

Cells were transformed by electroporation, transferred to sterile tubes containing 2 ml sterile LB and shaken at 37°C for 2 hours. Transformants were plated on nutrient agar supplemented with kanamycin and incubated overnight. Candidates were screened using primers D66220 and D66221, that were designed to flank the *narL* gene, and mutants were identified by size. Confirmed mutations were transferred back into the original strain, MG1655, and strains JCB387 and JCB3911 (JCB387Δ*fnr::Cm*^{R}), by P1 transduction and selected for kanamycin resistance to eliminate the risk of secondary mutations introduced during mutagenesis. These strains were designated, DJS100, DJS101 and DJS102, respectively.

Transfer of chromosomal mutations using P1 phage transduction

E. coli phage P1 is able to infect bacteria and produce viral particles that contain host DNA in place of viral DNA. This host-DNA-containing phage can then transfect other bacteria and, through homologous recombination with the recipient chromosome, transfer genetic information This makes them ideal vectors for the transfer of chromosomal mutations containing selectable markers between strains. Chromosomal mutations in isogenic strains were transferred to compatible strains to create double and triple mutants, using P1 transduction and antibiotic selection. Donor mutants, carrying an antibiotic resistance marker, were aerated at 37°C overnight in 1 ml LB supplemented with 2 mM $CaCl_2$. The next day, the culture was freshened by removing 0.5 ml of the culture and adding 0.5 ml fresh LB (2 mM $CaCl_2$) and aerating at 37°C for 3 hours. During this time, Lennox agar was prepared, autoclaved, cooled to 58°C and supplemented with 0.2% (w/v) glucose and 2 mM $CaCl_2$. While still warm, 1 ml of this agar was removed and added to sterile test tube containing 2 ml of pre-warmed LB (2 mM $CaCl_2$), vortexed immediately and placed in a 30°C water bath. The remaining agar was then used to pour thick agar plates which were set but not dried. Stock P1 was serially diluted from 10^{-1} to 10^{-6} in LB (2 mM $CaCl_2$). Once donor bacteria were ready, 0.1

ml of diluted P1 was added to the 3 ml of agar in the water bath, followed immediately by 0.1 ml of donor bacteria. The tube was then vortexed immediately and poured onto one of the agar plates to create a soft overlay of agar. This process was repeated for all dilutions of P1 to ensure a phage titre number that will give effective transduction without excessive bacterial lysis. Plates were then incubated at 37°C overnight and P1 was harvested by adding 2 ml of sterile LB (2 mM $CaCl_2$) and homogenising the soft agar layer, containing grown bacteria. Bacterial slurry was mixed with 1ml of ice-cold chloroform in a cooled hand homogeniser. Once homogenous, the slurry was centrifuged at 8,000 g and the P1 containing supernatant was retained for transduction. The recipient bacterium was then aerated overnight in 1 ml LB ($CaCl_2$), after which time it was tipped into 20 ml of sterile LB (2 mM $Cacl_2$) and aerated at 37°C for 4 hours. When grown the recipient culture was separated into three sterile tubes, spun down to a pellet and re-suspended in 0.5 ml LB (2 mM $CaCl_2$). To these three tubes were added either: 0.1 ml Undiluted P1, 0.1 ml 10^{-1} P1 or nothing to the control tube. The cells were then incubated at 37°C to allow for efficient infection, before the addition of 1 ml of sterile MS medium. Cells were spun down, supernatant discarded and re-suspended in 4 ml sterile MS. This process was repeated 4 times to ensure excess P1 was washed away. Finally, pellets were re-suspended in 2 ml LB and aerated for 1 hour before being pelleted once more and re-suspended in 0.2 ml MS. The entire suspension was then plated onto a dry agar plate supplemented with appropriate antibiotics. Plates were incubated at 37°C overnight. After incubation candidates were selected and purified onto a second antibiotic plate, spread for single colonies, and grown overnight. Once grown the presence of the antibiotic resistance cassette was confirmed using PCR and primers designed specifically to flank the insertion site of the cassette in the target gene.

Preparation of competent cells of *Escherichia coli*

Calcium chloride competent cells were prepared as described previously (Maniatis, 1983). A single healthy colony was used to inoculate 1 ml of sterile LB in a test tube. This tube was aerated overnight at 37°C unless otherwise stated. A 100 ml conical flask containing 20 ml of sterile LB was then inoculated with 500 µl of the overnight culture and further aerated at 37°C for 3 to 4 hours. The bacteria were then separated in to two sterile centrifuge tubes and pelleted by centrifugation at 8, 000g for 3 min. The supernatant was discarded and the pellet re-suspended in 2.5 ml of ice-cold, sterile 0.1 M $CaCl_2$. Bacteria were then stored on ice for at least 20 min. before being pelleted and re-suspended in a 100 µl volume of ice cold $CaCl_2$. Competent bacteria were used for transformation straight away or stored in sterile microfuge tubes at -80°C until needed. Calcium chloride competent cells were used for transformations involving high-copy number plasmids with a high transformation frequency. For transformations involving low-copy number plasmids with low transformation efficiency, a modified protocol using rubidium chloride, available at www.Qiagen.com, was used. In this protocol cells were grown to an OD_{650} of 0.5, cooled on ice for 10 min and pelleted in two 10 ml falcon tubes by centrifugation. The supernatant was carefully discarded and the pellets were resuspended in 3 ml per tube of ice-cold buffer TFB 1. The buffer TFB1 consisted of 100 mM RbCl, 50 mM $MnCl_2$ 30 mM potassium acetate, 10 mM $CaCl_2$ and 15% (v/v) glycerol, adjusted to pH 5.8 and filter sterilised. The resuspended cells were incubated on ice for a further 90 min, collected by centrifugation and the supernatant was discarded. Pellets were carefully resuspended in 1 ml ice-cold TFB2, which consisted of 0.1 mM $CaCl_2$ and 15% (v/v) glycerol, and separated into aliquots of 100-200 µl. Cells were 'snap frozen' in liquid nitrogen and stored at -80°C until needed.

Transformation of *Escherichia coli* with plasmid DNA

Competent cells were transformed with plasmid DNA by adding 1 to 3 µl of plasmid DNA to a 50 µl volume of competent cells and incubated on ice for 1 hour. Cells were then

heat shocked at 42°C for 2 min. to facilitate the uptake of DNA, followed by the immediate addition of 1 ml of sterile LB. Transformation mixtures were transferred to a sterile test tube and incubated at 37°C for one hour with vigorous shaking to allow expression of selective genes such as antibiotic resistance. The transformation mixture was then plated on agar plates supplemented with appropriate selective antibiotics.

Deletion of 'Datsenko and Wanner' mutant antibiotic resistance cassette using pCP20

All of the plasmids that are required to utilise this technique were kindly donated by Professor B. Wanner (Purdue University, Indiana). The antibiotic resistance gene carried by the strain to be cured was eliminated through transformation with the plasmid pCP20, which has a temperature sensitive origin of replication and encodes the FLP recombinase. Strains were transformed, as standard, but were selected on ampicillin containing plates grown at 30°C. Suitable candidates were selected, purified and re-grown at 42°C overnight to inhibit pCP20 replication and thus eliminate it from the bacterium. After heat treatment single colonies were replica plated to test for the elimination of chromosomal cassette and plasmid encoded ampicillin resistance.

DNA and RNA techniques

Agarose gel electrophoresis of DNA or RNA

DNA fragments of greater than 500 bp were separated by agarose gel electrophoresis. Agarose gels were prepared by dissolving 0.8% agarose (m/v) in TBE buffer and boiling briefly in a microwave. Gels were formed from liquid agarose using standard gel/well forming equipment and allowed to cool and set for 5 min. DNA/RNA samples were mixed in a 5:1 ratio with gel loading buffer and loaded into the gel. Gels were run in 1XTBE buffer supplemented with 0.5 μg ml^{-1} ethidium bromide at 100 V for 20-50 min. For analysis of RNA all agarose, sample buffers and TBE were prepared in DEPC treated water. Gels were visualised using a UV-transilluminator.

Polyacrylamide gel electrophoresis of DNA

DNA fragments smaller that 500 bp were analysed using polyacrylamide gel electrophoresis. Polyacrylamide gels contained 7.5% (w/v) stock acrylamide, 4% glycerol and 1 x TBE, and were polymerized by adding 0.01 volumes of 10% (w/v) ammonium persulphate and 0.001 volumes TEMED (N,N,N′,N′-Tetramethylethylenediamine). DNA samples were mixed in a 5:1 ratio with sample loading buffer and loaded onto the polyacrylamide gel. Gels were run in 1 x TBE at 30 mA for 20-30 min. then stained in a 0.5 μg ml^{-1} solution of ethidium bromide and visualised using a UV-transilluminator.

Sequencing polyacrylamide gel electrophoresis

For analysis of DNA footprint reactions and primer extension reactions, samples were seperated on thin denaturing 6% polyacrylamide sequencing gels (40 cm x 30 cm x 0.4 mm), prepared using the SequaGel sequencing system following the manufacturer's instructions. Gels were pre-run prior to loading for 2 hours at 60 W in 1 x TBE buffer. DNA samples were denatured at 95°C for 5 min in loading buffer before being loaded on the gel and run in 1 x TBE at 60 W for ~2 hours. Gels were fixed using a solution of 10% (v/v) acetic acid and 10% (v/v) methanol for 10 min, transferred to filter paper and dried under vacuum at 80°C for 30 to 40 min. Dried gels were visualised by exposing to a Fugi imaging phosphor screen, scanned using a Bio-Rad Molecular Imager FX and analysed using QuantityOne software (BioRad).

Phenol-chloroform extraction

In order to remove protein contamination, DNA samples were treated with an equal volume of phenol-chloroform, vortexed for 15 seconds and centrifuged for 3 min. The aqueous layer that contains the DNA was transferred into a clean tube. Where the volume of DNA sample was less than 200 μl, a volume of water equal to that of the aspirated aqueous layer was added to the phenol-chloroform for back-extraction, and the tube was vortexed for 15 seconds

and centrifuged for 3 min. The second aqueous layer was extracted and combined with the aqueous layer from the first extraction.

Ethanol precipitation of DNA

A 10% volume of 3 M sodium acetate (pH 5.2) and 3 volumes of ice cold 100% ethanol were added to the DNA sample and the solution was incubated at -20°C for 30 min. or longer. After 15 min. of centrifugation at 4°C, the supernatant was removed and the pellet was washed in 1 ml of ice cold 70% ethanol and centrifuged for 10 min. The supernatant was removed and the pellet was dried for 10 to 15 min. in a vacuum dryer. The DNA pellet was resuspended in 10 to 50 µl of sterile distilled water.

DNA purification using a QIAquick PCR purification kit

PCR products or restriction digestions were purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions.

Extraction of DNA from agarose gels using QIAquick gel extraction kit

DNA bands were excised from 0.8% agarose gels using a sterile scalpel under UV illumination. DNA was eluted using a QIAquick gel extraction kit following manufaturer's instructions.

Small scale extraction of plasmid DNA using QIAprep spin miniprep kit

An overnight culture of a strain carrying the desired plasmid was grown in 2 ml of LB supplemented with appropriate antibiotics. Plasmid DNA was then extracted form the culture using the QIAprep miniprep spin kit following the manufacturer's instructions. For low copy number plasmids, such as pRW50, 20 µl of elution buffer, pre-warmed to 50°C, was used to elute DNA from the column.

Construction of the *Eco*RI/ *Hin*dIII *yeaR100* promoter fragment by polymerase chain reaction

The intergenic region between *yeaR-yoaG* and the upstream gene *leuE* (*yeaS*) was amplified from MG1655 chromosomal template using colony PCR and primers, DSpyeaRfor and DSpyeaRrev (table 2.3). These primers introduced an *Eco*RI site and a *Hind*III site at the 5′ and 3′ end of the fragment, respectively. DNA products were purified using agarose gel electrophoresis on a 0.8% agarose gel and extracted using a Qiagen gel extraction kit following manufacturer's instructions. Purified PCR products were digested with *Eco*RI and *Hin*dIII, purified using the QIAGEN PCR purification kit and quantified by gel electrophoresis on a 0.8% agarose gel. The PCR fragment was also ligated directly into a T-easy (promega) vector and transformed into supplied super competent cells following manufacturer's instructions.

Cloning of promoter fragments into pRW50

Promoter fragments were prepared by PCR as described above, digested with *Eco*RI and *Hin*dIII, purified by gel electrophoresis and extracted. Vector DNA was isolated from a transformant using a Qiagen mini-prep kit. Purified plasmid was digested with *Eco*RI and *Hin*dIII, treated with alkaline phosphatase and purified by phenol/chloroform extraction and ethanol precipitation. Insert DNA was ligated into *Eco*RI-*Hin*dIII digested vector using T4 DNA ligase for 5 hours or overnight at 10°C. A 5 µl aliquot of the ligation mixture was used to transform competent cells prepared by the rubidium chloride method, as described, and selected on MacConkey-lactose agar supplemented with tetracycline. Candidates were screened for the presence of an insert by *Eco*RI/ *Hin*dIII digest and PCR using primers that flank the cloning linker. Candidates that showed a positive result for an insert were sequenced by the functional genomics centre operated by the University of Birmingham.

Purification of total RNA using the RNeasy Mini kit (Qiagen)

An overnight culture of the strain of interest was grown in 5 ml of LB medium supplemented with appropriate antibiotics. A 100 ml culture of MS medium supplemented with

5% LB, 0.4% glycerol and 20 mM nitrate was inoculated with 1 ml of the overnight culture and grown at 37°C without shaking until an OD of 0.4-0.5 was reached. At this point three 2.5 ml aliquots of culture were mixed with 4.5 ml of RNA later (Ambion), incubated at room temperature for 5 min. and pelleted by centrifugation for 20 min. at 4000 rpm. The supernatant was decanted and the pellets were stored at -80°C until required. For RNA extraction, all pellets were resuspended in 200 µl of DEPEC-TE buffer containing 40 µg ml^{-1} lysosyme and incubated for 15 min. at room temperature. RNA was extracted from the cell lysates according to the manufacturer's instructions.

To remove all DNA contamination, RNA preparations were treated with 5 µl of Turbo DNase buffer and 1 µl Turbo DNase (Ambion) and incubated at 37°C for 30 min. After this time 6.1 µl DNase inactivating reagent was added and samples were incubated for a further 2 min. at room temperature. Samples were centrifuged for 2 min. at 10, 000 rpm and the supernatant was transferred to a fresh tube. RNA concentration was determined using an Eppendorf Biophotometer. RNA samples were stored at -80°C.

Polymerase chain reaction

Polymerase chain reaction is used to replicate DNA between two short oligonucleotide primers using a thermostable DNA polymerase.

Primers were synthesised by Alta Biosciences (University of Birmingham) and are listed in table 2.3. Primers were supplied as a dry solid and were subsequently resuspended in sterile distilled water to a stock concentration of 100 µM prior to use. For PCR reactions, Invitrogen supermix PCR mix was used according to manufacturer's instructions. If plasmid DNA was used as a template, 3 µl of a 1 in 50 dilution of a mini-prep sample was used. For colony PCR a single colony was picked from an agar plate and resuspended in 100 µl of sterile distilled water. This suspension was then heated to 95°C for 10 min., centrifuged for 2 min. and 5 µl of the supernatant was used as a template. Each PCR reaction contained 40 µl PCR

supermix, 1 µl of each primer at 10 µM concentration, 5 µl of template and 4 µl sterile distilled water.

The PCR reaction consists of three stages. The first stage uses high temperature to melt the template DNA and separate the two strands (denaturation). The second stage involves cooling the reaction to a temperature optimal for primer binding (annealing). Thirdly the temperature is increased to the optimal temperature for DNA polymerase activity and the template strand is replicated (elongation). This cycle is repeated 30 times. For each reaction the optimal temperature for primer annealing was specific for each set of primers. To calculate the annealing temperature of each oligonucleotide the following equation was used. T_m= 64.9 + (G% + C%)*0.41-600/n, where G and C are the percentages of guanidine and cytosine in the oligonucleotide and n is the total length in nucleotides of the primer. For Invitrogen supermix polymerase the elongation temperature was 72°C. A typical reaction programme for amplification of a 1 kb product using primers with a 56°C annealing temperature would be as follows: Step one, 95°C for 30 s for denaturing, 30 s at 54°C for primer annealing and 90 s at 72°C for extension, repeated for 30 cycles. Step two would consist of a single cycle of 95°C for 30s for denaturing, 30 s at 54°C for primer annealing and 10 min. at 72°C for final extension.

DNA sequencing

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

QuickChange site-directed mutaganesis

Mutations were made in plasmid DNA using the QuickChange site directed mutagensis kit (Stratagene). This method involves the use of inverse-PCR to generate plasmid DNA with the desired mutations incorporated. Oligonucleotides are designed to anneal to both strands of the plasmid DNA in the region to be mutagenised. The site to be mutagenised is incorporated in the primer surrounded by 10 to 50 nt of specific sequence that will anneal to the plasmid template. A PCR reaction with a total volume of 50 µl was set up to contain 10% (v/v) reaction buffer, 5-50 ng of plasmid template, 125 mg of each mutagenic oligonucleotide primer, 1 µl of dNTP mixture and 1 µl of Pfu Turbo DNA polymerase (supplied in the kit). The PCR reaction was designed to the following parameters. Step one a single cycle of 95°C for 30 s for denaturing followed immediately by 18 cycles of 95°C for 30s for denaturing, 60 s at 54°C for primer annealing and 72°C for 'X' s, where x is equal to 2 min. per kilobase of plasmid template to be replicated. In each cycle the first step denatures the plasmid template, the second step allows oligonucleotide annealing and the third step allows for the entire plasmid to be copied, incorporating the mutagenic primers into the growing strand.

Following the PCR reaction, the reaction mixture contained a mixture of non-mutagenised template DNA and newly synthesised mutagenised plasmid. The reaction mixture was treated with the enzyme *Dpn*I, which specifically digests the parental methylated DNA but does not digest the newly synthesised non-methylated plasmid, containing the desired mutation. *E. coli* strain, XL1-Blue ultracompetent bacteria, were then transformed with the treated DNA and the resultant transformants were selected and purified on agar plates containing the appropriate antibiotics. All mutagenic primers used in this study are listed in table 2.6.

Restriction digest of DNA

Restriction enzymes were used to cut DNA at specific target sites according to the manufacturer's instructions. DNA was mixed with one unit of restriction enzyme and the

manufacturer's buffer, appropriate for each enzyme. The restriction mixture was incubated at 37°C for two hours.

Treatment of restriction digests with calf intestinal alkaline phosphatase (CIAP)

This enzyme helps to limit plasmid re-circularization during ligation by removing the 5′ phosphate group from the digested DNA molecule. After digestion with restriction enzymes, vector DNA was treated with 10 units of CIAP and incubated for 1 hour at 37°C. CIAP was also used to remove the 5′ phosphate group from promoter fragments to be used in electromobility shift assays and footprinting experiments following the same protocol.

T4 DNA ligase

This enzyme was used to ligate two pieces of digested DNA together by the formation of a phosphodiester bond between the 5′ phosphate of one strand and the 3′ hydroxyl group of the other strand. DNA strands to be ligated were incubated at 10°C for 8 hours or overnight with 1 unit of T4 DNA ligase and the appropriate buffer (supplied with enzyme).

Radio-labelling of promoter DNA

Promoter fragments to be used in electromobility shift assays were radio-labelled using T4 polynucleotide kinase (NEB) and radioactive γ-phosphate from [γ^{32}P]-ATP (Amersham). Promoter fragments were prepared by digestion with appropriate restriction enzymes, treated with CIAP to remove the 5′ phosphate group of the digested fragment and purified by agarose gel electrophoresis and gel extraction. A reaction mixture containing 50 ng of purified promoter DNA, 1 µl of stock [γ^{32}P]-ATP, 1 unit of T4 polynucleotide kinase, and 2 µl of appropriate buffer in a total volume of 20 µl was incubated at 37°C for 30 min. Unincorporated nucleotides were removed from the reaction mixture using a G50 Sephadex spin column (Bio-Rad).

Protein purification and analysis

Denaturing gel electrophoresis of proteins

Protein samples were separated and analysed using NuPAGE 12% Bis-Tris pre-cast gels (Invitrogen). Cells were grown in conditions of interest, to an OD_{650} ~0.5, at which point 500 µl of culture was harvested by centrifugation at 13, 000 r.p.m for 1 min and resuspended in a volume of sample buffer 50 x the OD_{650} (i.e. for an OD of 0.5, 250 µl of sample buffer was added). Pellets were heated to 95°C for 5 min., vortexed for 10 s, centrifuged for 15 s at 13,000 rpm and loaded onto the gel. Gels were run following manufacturer's instructions in an XCell SureLock electrophoresis system (Invitrogen) in 1 x MES SDS running buffer (Invitrogen) at 140 V for approx 1 h. Gels were stained with either Coomasie stain or SilverQuest (Invitrogen) silver stain according to manufacturer's instructions. For Coomassie staining, gels were incubated in Coomassie stain solution for 30 min with constant agitation and de-stained for 1 hour with agitation in fast de-stain solution. Gels were soaked in shrink solution and dried between two sheets of cellophane.

Western blotting

Western blotting was used for semi-quantitative determination of specific cellular proteins such as NarL. Cultures were grown anaerobically in minimal medium supplemented with 20 mM fumarate, 20 mM TMAO, 2.5 mM nitrite and 0.4% glycerol as a carbon source. When cultures reached mid-exponential phase, the exact OD_{650} was taken and used to calculate the amount of sample buffer needed for lysis (OD_{650} x 50) before samples were harvested by centrifugation at 13, 000 rpm for 1 min. Pellets were resuspended in the appropriate amount of sample buffer, heated to 95°C for 5 min. then re-centrifuged for 1 min. at 13,000 rpm. Samples were then diluted 10 or 100 fold in sample buffer and loaded on a NuPAGE 12% Bis-Tris pre-cast gels (Invitrogen). Gels were run following manufacturer's instructions in an XCell SureLock electrophoresis system (Invitrogen) in 1 x MES SDS running buffer (Invitrogen) at

140 V for approximately 1 h. Gels were not stained at this point but were soaked for 10 min. in transfer buffer. Meanwhile, a piece of Hybond-ELC nitrocellulose membrane (Amersham scientific) and 8 pieces of filter paper, the same size as the gel, were soaked for 5 min. in distilled water then 5 min. in transfer buffer. Care was taken at all times not to touch the gel or membrane and forceps were used for all handling procedures. The gel, membrane and pre-soaked filter paper were assembled in an XCell II blot module (Invitrogen) according to manufaturer's instructions and all bubbles were carefully removed using a clean glass Pasteur pipette. The transfer module was filled with transfer buffer and run at 30 V for 3 hours at room temperature. Following transfer, the nitrocellulose membrane was washed twice in 25 ml of 1x TBS for 5 min and once in 25 ml blocking buffer for 1 hour at room temperature with constant shaking. The filter was washed three times for 5 min. in 25 ml of TBS/T with vigorous shaking then incubated overnight in a sealed polythene bag in 10 ml of blocking buffer containing an appropriate dilution of the primary anti-body (for mouse anti-NarL: 1 µl in 10 ml blocking buffer). After incubation, the membrane was washed 4 times for 5 min. in 25 ml TBS/T then incubated for 1 hour in a sealed bag in 10 ml blocking buffer containing an appropriate amount of the secondary HRP-conjugated antibody. The membrane was washed three times for 5 min. with 25 ml TBS/T with constant shaking, drained of excess solution and placed protein side up on a piece of polythene wrap. Secondary antibody binding was visualised using the Amersham ECL Plus Western blotting detection reagents (GE Healthcare), as described in the manufacturer's protocol, and imaged using autoradiography film and an X-ograph imager operated by the functional genomics suite at the University of Birmingham.

Protein preparations

The NarL-MBP protein fusion (maltose-binding protein) was purified and kindly donated by Dr Douglas Browning (University of Birmingham) following the method described previously (Li *et al.*, 1994). Prior to use in all experiments the mature NarL was used after

MBP moiety had been cleaved from MBP-NarL using factor Xa (NEB). Purified NarL and NarP proteins were phosphorylated prior to use by incubating the protein with 500 μM acetyl phosphate for 30 mins at 37°C. The D154A FNR protein was prepared, purified and kindly donated by Dr David Lee following the method described previously (Wing *et al.*, 2000).

In vitro techniques

Electro-mobility shift assay (EMSA)

For each EMSA, a known concentration of either NarL or FNR protein was diluted in Hepes buffer (1 μg ml^{-1} BSA) and incubated for 30 min. at 37°C with 0.2 ng of P^{32}-labelled promoter DNA, 1 μl FNR binding buffer, 1 μl of 50% (v/v) glycerol, 5 μg of BSA and 250 ng of herring sperm DNA in a final volume of 10 μl. In gel retardation experiments involving NarL or NarP, the proteins were pre-phosphorylated by incubating the protein for 30 min in 50 mM acetyl phosphate (final concentration). After incubation, DNA-protein complexes were run in 0.25 X TBE (Tris/borate/EDTA; 1 x TBE = 45 mM Tris/borate and 1 mM EDTA) on a 6% polyacrylamide gel containing 2 % glycerol. Gels were run at a constant voltage of 12 V cm^{-1} gel for approximately 2 hours.

Following electrophoresis the gel was fixed with a solution containing 10% (v/v) methanol and 10% (v/v) acetic acid for 10 min. and vacuum dried on Whatman 3MM paper for 30 min. The radio-labelled DNA fragments were visualised using a phosphor screen (Fuji) and scanned using Molecular Imager FX and QuantityOne software available at the functional genomics suite at the University of Birmingham.

Mapping of transcription start site by primer extension

The transcription start site of *Eco*RI-*Hin*dIII promoter fragments, cloned into pRW50, was determined using the primer, D49724 (table 2.3), which anneals 83 bp downstream of the *Hin*dIII cloning site of pRW50. Primer D49724 was end-labelled by mixing 0.5 μl of 100 μM primer with 1 μl of [γ^{32}P]-ATP (Perkin Elmer) and 1 μl of T4 polynucleotide kinase (NEB) in a

final volume of 20 µl polynucleotide kinase buffer (NEB). After 30 min. incubation at 37 °C, the reaction was heated to 68°C for 10 min. to deactivate the enzyme.

For preparation of mRNA, cultures of strain, JCB387, transformed with the plasmid, pYEAR100, were grown at 37°C without shaking in 100 ml of defined medium, in a 100 ml conical flask, supplemented with 0.4% (v/v) glycerol and 20 mM $NaNO_3$. At mid-exponential growth phase, cells were mixed with RNA-later (Ambion), harvested by centrifugation and stored at -80°C. The mRNA was extracted using an RNAeasy kit (QIAGEN) following manufacturers instructions and the quality and concentration of mRNA was determined using a spectrophotometer. Between 20 and 30 ng of purified RNA was mixed with 1 µl of ^{32}P-labelled primer and ethanol precipitated by adding 10% volume of 3 M sodium acetate (pH 7) and 2.5 volumes ice cold 100% ethanol. Samples were incubated at -80°C for 10 min. to precipitate the RNA before the sample was centrifuged at 13, 000 rpm for 10 min. at 4°C and washed in 1 ml of 70% ethanol. The RNA primer mix was then pelleted by centrifugation at 13, 000 rpm. at 4°C for 5 min. before being vacuum dried and resuspended in 30 µl of hybridization buffer by vortexing for 5 min. The hybridization mixture was incubated at 75°C for 15 min. then at 50°C for 3 hours to anneal the primer to the RNA template. After incubation, 75 µl of 100% ethanol was added, the solution was votexed and were incubated at -80°C overnight. The annealed primer/ RNA was pelleted by centrifugation at 13,000 rpm for 10 min., washed with 1 ml of 70% ethanol and centrifuged at 13,000 rpm for 5 min. before being vacuum dried and resuspended in 30 µl RNase-free water. To this sample, 10 µl of 5 x reverse transcriptase buffer (Promega), 1 µl 50 mM DTT, 5 µl 10 mM dNTPs, 2.5 µl AMV reverse transcriptase (Promega) and 0.6 µl of Rnasin (Promega) were added and the reverse transcription reaction was incubated for 1 h at 37°C. The reaction mixture was heated to 72°C for 10 min. to inactivate the enzyme, before 1 µl of 10 mg ml^{-1} RNase was added and the sample was incubated for 30 min at 37°C. After this time, 5 µl of 4 M ammonium acetate pH 4.8 and 125 µl cold 100% ethanol were added to precipitate the cDNA, and the solution was centrifuged at 13,000 rpm at

4°C for 10 min. The pellet was washed with 1 ml of cold 70% ethanol and centrifuged for a further 5 min. at 13,000 rpm at 4°C. The pellet was then completely dried under vacuum and resuspended in 4 µl of stop solution from the T7 sequencing kit (USB)

From this reaction mixture, 3 µl of each primer extension reaction was loaded onto a 6% acrylamide sequencing gel, together with 3 µl each of the G, A, T and C sequencing reactions prepared using the USB T7 sequencing kit as described in the next section. The gel was run at 60 W for approximately 2 h, then fixed for 10 min. in a 10% methanol, 10% acetic acid (v/v) solution. The gel was vacuum dried or 30 min. and exposed on a phosphor-screen (Fugi imaging) for 4 hours or overnight. The image on the phosphor-screen was visualised using Molecular Imager FX and QuantityOne software (Bio-Rad) available at the functional genomics suite at the University of Birmingham.

Dideoxy chain termination DNA sequencing

DNA sequencing reactions, used for the size calibration of primer extension gels, were prepared by dideoxy sequencing reactions using the USB T7 sequencing kit. Template plasmid (pSR) DNA was prepared using a Qiagen Miniprep kit. To prepare single stranded plasmid template, 15 µl of miniprep plasmid DNA was mixed with 15 µl of sterile water and 3 µl of 2 M sodium hydroxide and incubated at room temperature for 15 min. The single-stranded DNA was precipitated by adding 3 µl of 5 M ammonium acetate, pH 4.8, and 75 µl of ice cold ethanol, incubating for 15 min. at -20°C and centrifuging at 13,000 rpm for 15 min. The pellet was washed with 1 ml of 70% ice-cold ethanol and centrifuged at 13,000 rpm at 4°C for a further 5 min. The pellet was dried under vacuum and resuspended in 10 µl of sterile water. The 10 µl single stranded DNA template was mixed with 2 µl 4 µM sequencing primer, D36245, and 2 µl annealing buffer (USB T7 sequencing kit), incubated for 20 min. at 37°C and cooled to room temperature for 10 min. Sequencing reactions were continued according to manufacturers instructions using [α^{32}P]-dATD (Perkin Elmer) and the 'read short' conditions

described in the USB T7 sequencing kit. In a modification to the manufacturer's instructions, 12 µl of stop solution was added to terminate the reaction and sequencing reactions were stored at -20°C.

DNase I footprinting

A master reaction mix was set up containing, per footprinting reaction, 0.2 µl of a 400 nM radiolabelled fragment, 2 µl of binding buffer, 1 mg ml^{-1} BSA in a total volume of 20 µl sterile distilled water. This solution was aliquoted into separate micro-centrifuge tubes. The appropriate protein dilution was pipetted into each tube and mixed by 'flick-spinning' before being incubated for 20 min. at 37°C. DNase I was added directly to the sample and mixed by gentle stirring for 15 seconds. The reaction was stopped by the addition of 200 µl DNase I stop solution and thorough mixing. Digested DNA fragments were extracted by the addition 200 µl phenol, vortexed for 15 seconds and centrifuged at 13,000 rpm for 2 min. Approximately 190 µl of the aqueous layer was removed and placed in a fresh micro-centrifuge tube. This sample was treated with 1 µl of 20 mg ml^{-1} glycogen and 400 µl of ice-cold 100% ethanol, mixed well and incubated at -80°C for 15 min. The DNA was pelleted by centrifugation for 15 min. at 13,000 rpm, washed with 1 ml of 70% ethanol and re-centrifuged for 10 min. at 4°C. All solutions were treated as radioactive waste and discarded appropriately. The pellet was dried under vacuum at 45°C and resuspended in 8 µl of DNase I loading buffer. All samples were heated to 90°C for 2 min. prior to loading 4 µl of each sample onto a denaturing polyacrylamide gel (6% acrylamide and 8% urea) and calibrated with the products of a Maxam-Gilbert 'G+A' sequencing reaction. Gels were run at 60 W for approximately 2 hours, fixed for 10 min. in a solution of 10% (v/v) methanol and 10% (v/v) acetic acid in distilled water and visualised by exposure to a phosphor-imaging screen (Fuji). Footprint images were analysed using Molecular Imager FX and QuantityOne software (Bio-Rad) available at the functional genomics suite at the University of Birmingham.

Potassium permanganate footprinting

Potassium permanganate reaction mixtures were set up with the following constituents per reaction: 0.2 µl of end-labelled fragment; 2 µl of 10X hepes buffer, 1 µl 10 mg ml^{-1} BSA; 1 µl 20 mM DTT and the appropriate concentration of protein(s) of interest to a final volume of 20 µl. RNA polymerase was always added with a final concentration of 50 nM diluted in transcription buffer. Once the addition of protein had been made, reactions were incubated for 30 min. at 37°C before the addition of 1 µl of freshly prepared 200 mM $KMnO_4$ per tube and a further incubation for 4 min. at 37°C. The reaction was stopped with the addition of 50 µl of potassium permanganate stop solution and thorough mixing. Sterile water was added to make the total volume up to 200 µl, which was then added to an equal volume of phenol-chloroform and mixed well. The mixture was centrifuged for 3 min. at 13,000 rpm and the aqueous layer was removed and placed in a fresh microcentrifuge tube. DNA was extracted by ethanol precipitation by adding 400 µl of ice-cold 100% ethanol and 1 µl of 20 mg ml^{-1} glycogen and incubating at -70°C for 15 min. DNA was pelleted by centrifugation, washed with 1 ml of ice-cold 70% ethanol and re-pelleted. The pellet was dried under vacuum and re-suspended in 40 µl of 1 M piperidine. The reactions were incubated at 90°C for 30 min. before the addition of 10 µl of 3M sodium acetate (pH 5.2), 1 µl of 20 mg ml^{-1} glycogen and 70 µl sterile distilled water. DNA was precipitated by standard ethanol precipitation and the pellet was resuspended in 8 µl of loading buffer. Fragments were separated by PAGE on a standard 6% Sequa-gel and were calibrated with the products of a Maxam-Gilbert 'G+A' sequencing reaction. Gels were run at 60 W for approximately 2 hours, fixed for 10 min. in a solution of 10% (v/v) methanol and 10% (v/v) acetic acid in distilled water and visualised by exposure to a phosphor-imaging screen (Fuji). Footprint images were analysed using Molecular Imager FX and QuantityOne software (Bio-Rad) available at the functional genomics suite at the University of Birmingham.

Preparation of Maxam-Gilbert 'G+A' sequencing reaction

Radiolabelled *Aat*II-*Hin*dIII promoter fragments were used to create 'G+A' sequencing ladders for the calibration of DNA footprinting gels. 3-4 µL of radiolabelled DNA were diluted with sterile distilled water to a working volume of 12 µl, treated with 50 µl formic acid, and incubated at room temperature for 90 s. The reaction was stopped by adding 200 µl of 0.3 M sodium acetate (pH 7.0) and 700 µl of ice-cold 100% ethanol and DNA was purified by ethanol precipitation and dried under vacuum as described earlier. DNA was resuspended in 100 µl 1 M piperidine, vortexed well and incubated at 90°C for 30 min. DNA was purified by ethanol precipitation, resuspended in 20 µl of denaturing gel loading buffer, and heated to 90°C before loading 2 µl onto footprinting gels. All stages involving piperidine were carried out in a fume hood.

DNA sampling experiments: Strain preparation and DNA-protein isolation

E. coli strain MG1655 encoding a 3 x FLAG-tagged LacI was co-transformed with pDJS901 and pACBSR-DL1. A single colony of transformant was used to inoculate 1 ml of LB, which was grown at 37°C with shaking for 2 h then added to 2 l sterile pre-warmed minimal medium in a 2 l conical flask, supplemented with 20 mM fumarate, 20 mM TMAO, 2.5 mM nitrite and 0.4% glycerol as a carbon source. This culture was grown at 37°C without shaking. When cultures reached an OD_{650} of 0.6, 0.4% l-arabinose was added to induce expression of the I-*Sce*I meganuclease and the bacteriophage lambda Gam protein. Cultures were allowed to grow for 40 min post-induction before being harvested by centrifugation (15 min, 8000 r.p.m, room temperature). Cell pellets were resuspended in 20 ml of extraction buffer (50 mM Tris-Hcl (pH 7.5), 100 mM NaCl, 10% (v/v) glycerol, 2 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 200 µg ml^{-1} phenylmethylsulfonyl fluoride, 4 µg ml^{-1} pepstatin and a Roche diagnostics EDTA-free protease tablet) supplemented with 300 µg ml^{-1} RNAse and 400 µg ml^{-1} lysozyme. After 10 min incubation at room temperature, the mixture was cooled on ice and

sonicated for three periods of 30 s. Samples were centrifuged at 18,000 r.p.m for 20 min at 4°C to obtain a clear lysate.

To obtain DNA-protein complexes the lysates were mixed with 25 mg of Dynabeads (M270-epoxy; Invitrogen) that had been previously cross-linked to mouse anti-FLAG antibody (F3165, Sigma), and incubated on ice for 10 min. Antibody conjugated Dynabeads were kindly donated by Dr David Lee, University of Birmingham. Dynabeads were then collected with a magnet, washed 5 times with wash buffer (see materials and methods) in 2 ml unsiliconized tubes (Eppendorf). DNA-protein complexes were eluted from the anti-FLAG antibodies by thorough mixing of the beads with 500 µl of elution buffer see materials and methods), which was then separated from the beads with a magnet and removed to a fresh tube. Samples were dried under vacuum, dissolved in SDS-PAGE loading buffer containing 10 mM *tris*(2-carboxymethyl)phosphine–HCl (Sigma), heated at 95°C for 5 min and then alkylated by addition of 50 mM iodoacetamide (Sigma) for 30 min at room temperature. Proteins in the sample were resolved by SDS–PAGE in 4–12% gradient gels (Invitrogen), and visualized by SilverQuest^{TM} silver staining (Invitrogen). The relative size and intensity of bands in each sample were compared to each other and standard Invitrogen SeeBlue Plus 2 protein markers.

Excision of bands of interest, de-staining and trypsinisation

To identify proteins of interest in SDS-PAGE gels, gel slices of approximately 1 mm were excised, placed in clean eppindorf tubes and destained using the de-stain solutions included in the SilverQuest silver staining kit. Gel slices were dehydrated for 10 min in 100 µl of 100 % methanol, partially rehydrated in 100 µl 30% (v/v) methanol for 5 min and washed twice in 200 µl of ultrapure water. Gel slices were then washed three times for 10 min with 100 µl of wash solution containing 100 mM ammonium bicarbonate and 30% (v/v) acetonitrile. After the final wash gel slices were cut into small fragments (~1 mm^2) and washed in 100 µl ultra pure water for 10 min. and dried in a SpeedVac for 30 min. Gel slices were resuspended

in 50 µl of digestion buffer (50 mM ammonium bicarbonate) containing 5-10 ng/µl porcine trypsin and incubated overnight at 37°C. To extract peptides, gel slices were centrifuged at maximum speed and the supernatant was carefully removed to a fresh eppindorf tube. Pepetides were extracted by adding 20 µl of 50% (v/v) acetonitrile containing 0.1% (v/v) triflouroacetic acid, incubating at room temperature for 20 min. Extract from this step was combined with the supernatant obtained from centrifugation and the sample was concentrated to 4-5 µl in a SpeedVac. Samples were then sent for analysis on a Thermo-Finnegan FT-ICR mass spectrometer using a NanoMate chip-based electrospray system operated by the functional genomics suite at the University of Birmingham.

NarL-3xFLAG tagged protein pull-down

A single colony of each strain carrying the chromosomal-encoded NarL-3xFLAG tagged protein was grown in sterile LB for 2 hours at 37°C with constant shaking. This culture was used to inoculate 2 l of sterile pre-warmed minimal medium in a 2 l conical flask, supplemented with 20 mM fumarate, 20 mM TMAO, 2.5 mM nitrite and 0.4% glycerol as a carbon source. Cultures were grown at 37°C without shaking overnight or until an OD_{650} of 0.5 was reached. At this point cells were collected by centrifugation (15 min, 8000 r.p.m, room temperature). Cell pellets were resuspended in 20 ml of extraction buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% (v/v) glycerol, 2 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 200 µg ml^{-1} phenylmethylsulfonyl fluoride, 4 µg ml^{-1} pepstatin and a Roche diagnostics EDTA-free protease tablet) supplemented with 300 µg ml^{-1} RNAse, 20 µg ml^{-1} DNAse and 400 µg ml^{-1} lysozyme. Cells suspensions were lysed by sonification for 3 periods of 30 s and a clear lysate was obtained by centrifugation at 18,000 r.p.m for 20 min at 4°C. Lysates were then mixed with 25 mg of Dynabeads that had been pre-conjugated with anti-FLAG antibodies and NarL-FLAG was isolated and eluted by the same method described for DNA sampling experiments. Extracted NarL-FLAG protein was separated by SDS-PAGE, silver stained, excised and

digested with porcine trypsin as described for DNA sampling. Peptides were extracted by adding 20 µl of 50% (v/v) acetonitrile containing 0.1% (v/v) triflouroacetic acid, incubating at room temperature for 20 min. Extract from this step was combined with the supernatant obtained from centrifugation and the sample was concentrated to 4 to 5 µl in a SpeedVac. Samples were then sent for analysis on a Thermo-Finnegan FT-ICR mass spectrometer using a NanoMate chip-based electrospray system operated by the functional genomics suite at the University of Birmingham.

Chapter 3

Results

Studies on the physiological role of the genes of unknown function hcp and yeaR-yoaG and their implication in nitrosative stress management

Introduction

During its life as a gastro-intestinal pathogen, *E. coli* will often come into contact with reactive nitrogen species, such as nitric oxide and other secondary products, that are highly toxic to the bacterium. Nitric oxide is produced by macrophages, as part of the host innate immune response to damage invading bacteria, produced endogenously by *E. coli* when respiring nitrite, generated by other bacteria that share its ecological niche, and generated chemically from nitrite in an acidified environment (Corker and Poole, 2003; Crawford and Goldberg, 1998; Fang, 2004). Nitric oxide is highly reactive and can cause damage to many cell components including DNA, proteins and thiol-groups (Arnelle and Stamler, 1995; Kunisaki and Hayashi, 1979; Sedgwick, 1997; Taverna and Sedgwick, 1996; Weiss, 2006). Nitric oxide also forms adducts with iron-sulphur centres, which constitute essential co-factors of many enzymes, electron transfer chains and the sensing components of transcription factors (Cruz-Ramos *et al.*, 2002; D'Autreaux *et al.*, 2002; Justino *et al.*, 2007). Several transcriptomic studies have identified a number of genes that are expressed in reponse to the presence of nitric oxide or nitric oxide related molecules, generated by degradation of NO-releasing products (Constantinidou *et al.*, 2006; Flatley *et al.*, 2005; Justino *et al.*, 2005b; Mukhopadhyay *et al.*, 2004; Pullan *et al.*, 2007). A group of genes were identified by one of these studies as being upregulated by the presence of nitrate, which would be degraded via nitrite to generate nitric oxide, and were therefore implicated as candidate genes involved in nitrosative stress management. Two of the operons identified as being upregulated in conditions where nitrosative stress is likely were *hcp-hcr*, an operon encoding the hybrid cluster protein, Hcp, and its cognate NADH oxidoreductase, Hcr, as well as the operon of unknown function *yeaR-yoaG*. Both

of these operons were also identified as being regulated by the regulator of nitrosative stress, NsrR, which is thought to be a nitric oxide sensitive repressor (Bodenmiller and Spiro, 2006; Filenko *et al.*, 2007; Partridge *et al.*, 2009; Tucker *et al.*, 2008).

A possible role of the hybrid cluster protein in hydroxylamine reduction has previously been proposed. However, all reduction rates were calculated *in vitro* using purified Hcp (Wolfe *et al.*, 2002). The K_m of Hcp for the substrate hydroxylamine was shown to be high in physiologically relevant conditions (~40 mM), which is clearly irrelevant *in* vivo as a concentration of 1 mM is sufficient to cause cell death (data not shown). Hydroxylamine is produced by the nitrite reductases, NirB and NrfA, as an enzyme-bound reduction intermediate during the 6-electron reduction of nitrite to ammonia (Jackson *et al.*, 1981). However, whether this hydroxylamine escapes the active sites of the nitrite reductases is not known.

In order to investigate the possible roles of the hybrid cluster protein and the products of the operon of unknown function, *yeaR-yoaG*, in nitrosative stress management, the growth characteristics of knock-out mutants for each of the genes was investigated during growth in the presence of both hydroxylamine and nitric oxide.

Anaerobic growth of a *hcp* null mutant in alternative electron acceptors

To characterize the role of Hcp during anaerobic growth and nitrosative stress, a chromosomal *hcp-hcr* deletion mutation was created by replacing the coding region of *hcp-hcr* with a chloramphenicol resistance cassette using the lambda *red* method described previously (Datsenko and Wanner, 2000). The ability of this mutant to grow anaerobically in the presence of alternative electron acceptors was compared to the parental strain, RK4353 (fig.3.1). When grown in minimal medium supplemented with fumarate, TMAO, DMSO or nitrate, alternative electron acceptors that *E. coli* can respire in place of oxygen, the *hcp* null mutant showed a slight anaerobic growth defect in all conditions. The defect was most pronounced during growth with fumarate as terminal electron acceptor. Surprisingly however, the growth defect

Fig. 3.1

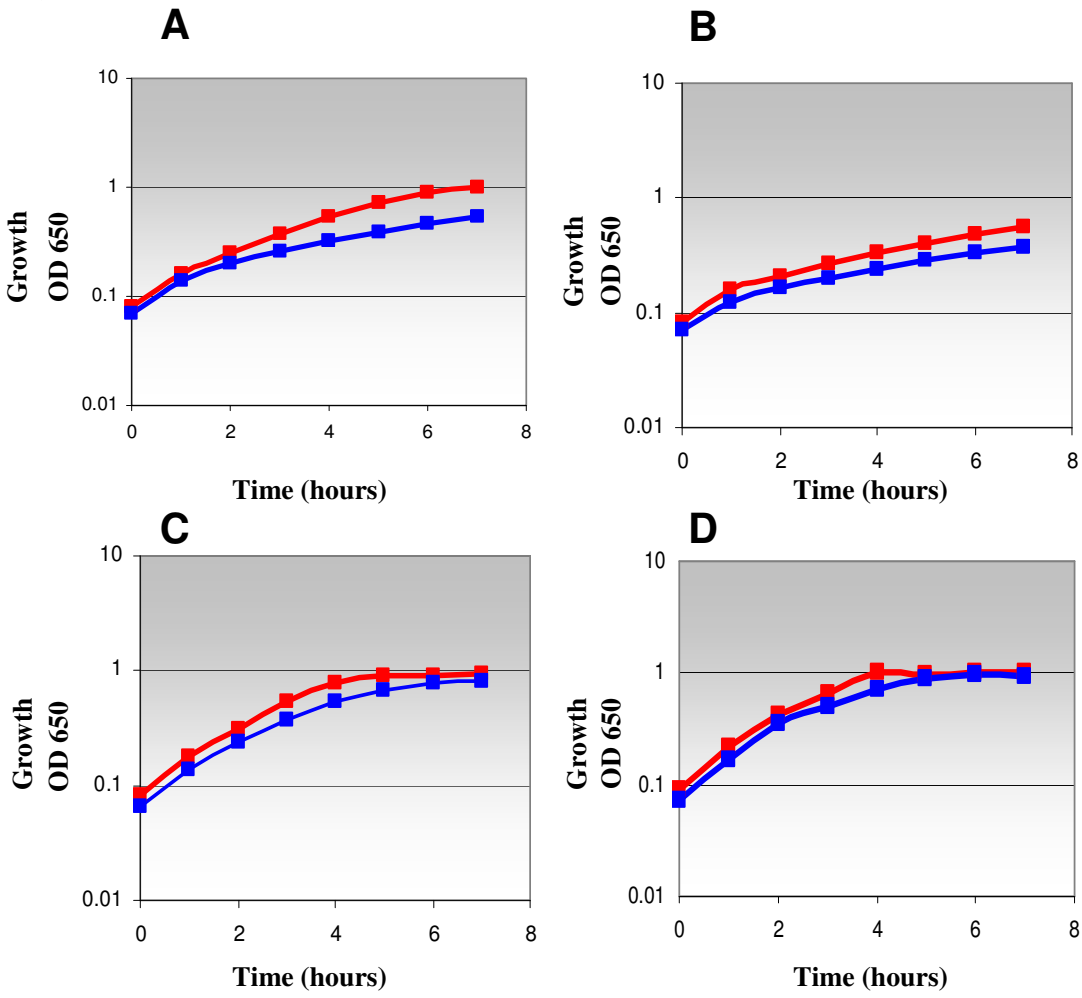

Figure 3.1 Anaerobic growth of the parental strain, RK4353, and a *hcp* null derivative, JCB5000, in the presence of alternative electron acceptors

An overnight culture of each strain was used to inoculate 100 ml of minimal salts medium supplemented with 5% LB, 0.4% glycerol and: **A**: 20 mM fumarate; **B**: 20 mM DMSO; **C**: 20 mM TMAO or **D**: 20 mM NO_3^-. The cultures were incubated without shaking at 37°C and the growth of the parental strain (RED) and the *hcp* null derivative (BLUE) was monitored for seven hours post inoculation. These data represent a typical result of three or more repeated experiments.

was least pronounced in the presence of nitrate (fig. 3.1 D), when toxic nitrogen species are most likely to be produced. Due to the growth defect being present in all conditions, even when reactive nitrogen species are unlikely to be produced (fumarate, TMAO and DMSO), the defect was not attributed to an inability of the *hcp* mutant to deal with any reactive nitrogen species present in the medium.

Nitric oxide inhibition of anaerobic growth rate in an *hcp* mutant

Previous transcriptomic investigations have identified a putative role for the nitric oxide sensitive repressor, NsrR, in regulation of *hcp-hcr* transcription, implicating an involvement of the gene products of this operon in nitrosative stress management. Therefore, the effect of nitric oxide on the growth of the *hcp-hcr* mutant, JCB5000, was compared with that of the parental strain, RK4353. Both strains were grown anaerobically in the presence of alternative electron acceptors, TMAO and fumarate, before being treated with 0, 10 or 40 μM nitric oxide. The effects of nitric oxide on the parental strain and *hcp* mutant were compared by the decrease of the exponential growth constant, μ, due to the mutation (fig 3.2). The greatest inhibition of the mutant in comparison to the parental strain was in the presence of 10 μM NO, where the growth rate of the mutant was inhibited by ~45% compared to 25% in the parental strain. The growth inhibition of the mutant by 40 μM NO was approximately equal to that of the parental strain, probably due to the fact that at this concentration of NO the parental strain was almost fully inhibited as well. This result indicated that Hcp may have some role in dealing with nitric oxide or a secondary product caused by nitric oxide toxicity.

The effect of hydroxylamine on the anaerobic growth yield of *E. coli*, with or without nitrite adaptation

To assess the effect of Hcp on the growth yield of *E. coli*, the parental strain, RK4353 and an *hcp-hcr* mutant, JCB5000, were grown anaerobically in the presence of 500 μM hydroxylamine (fig. 3.3). The addition of hydroxylamine caused a temporary growth inhibition of both strains. After 40 hours, growth of both strains was evident but the optical density of the

Fig. 3.2

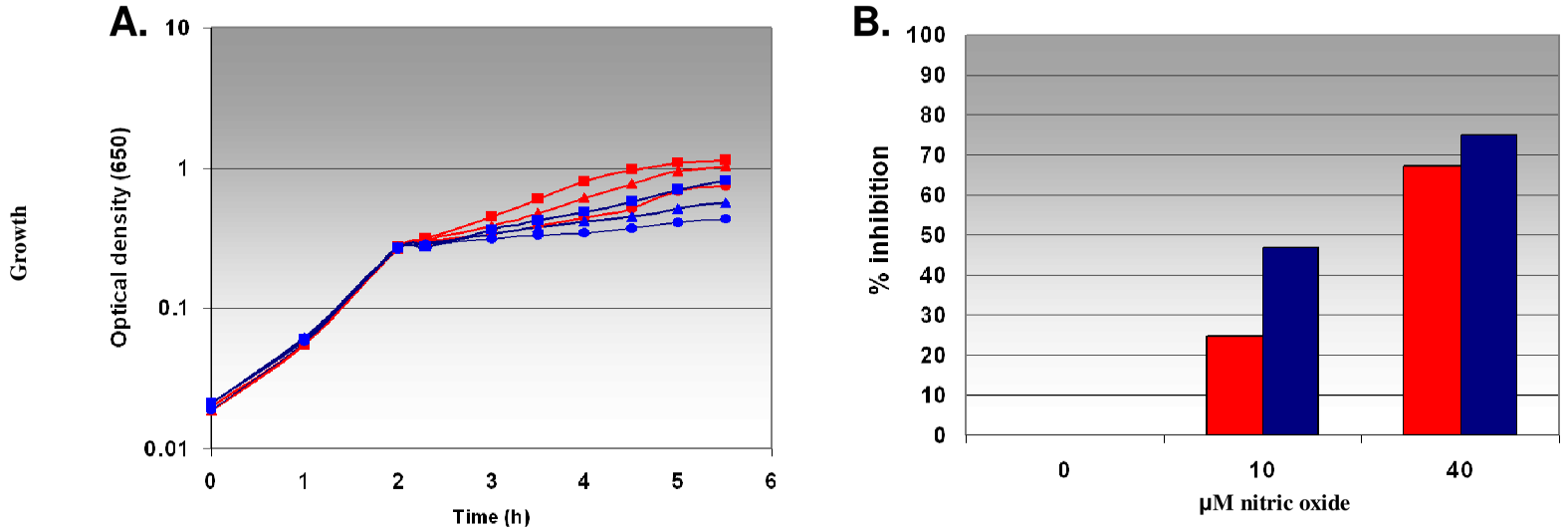

Fig. 3.2 Anaerobic growth inhibition of the parental strain, RK4353, and the *hcp* mutant, JCB5000 by nitric oxide saturated water

A. An overnight culture of the *hcp* mutant (blue) and the parent strain (red) was used to inoculate 100 ml of MS 5% LB supplemented with 0.4% glycerol, 20 mM TMAO and 20 mM fumarate in a 100 ml conical flask. At mid-exponential growth phase (OD_{650} ~0.3), cultures were pulsed with 0, 10 or 40 µM nitric oxide (filled squares, triangles and circles respectively). This pulse was repeated every 30 minutes for 3.5 hours. These data are representative of three or more independent experiments

B. The exponential growth constant, µ, was calculated for each culture and the percentage inhibition of µ in comparison to the untreated culture was determined. The value of µ was calculated from the gradient of the growth curve between 30 minutes and 1.5 hours after the first NO treatment. These data are representative of three or more independent experiments.

Fig. 3.3

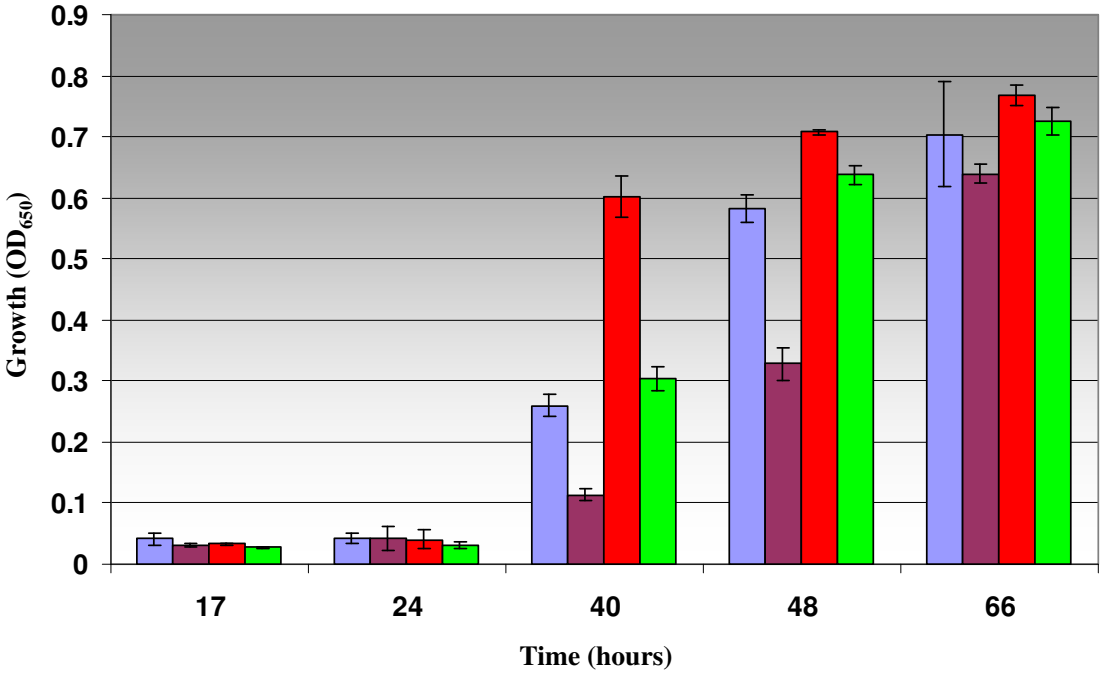

Fig. 3.3 Anaerobic growth yield of parental strain, RK4353, and the *hcp* mutant, JCB5000, after stress with 0.5 mM hydroxylamine with or without nitrite adaptation

In this and all subsequent experiments, unless otherwise stated, strains were pre-cultured overnight in LB medium at 37°C with aeration. A 5% inoculum of each strain was used to inoculate 10 ml of MS 5% LB supplemented with 0.4% glycerol, 20 mM TMAO, 20 mM fumarate and 0.5 mM hydroxylamine (blue and burgundy). Half of the tubes were also supplemented with 2.5 mM nitrite (red and green). The growth of RK4353 (blue and red) and the *hcp* null derivative, JCB5000 (burgundy and green), was monitored for 66 hours.

parental stain was approximately double that of the mutant. Similarly, for all time points up to 66 hours of growth, growth yield of the *hcp* mutant was lower than that of the parental strain, indicating greater sensitivity of the mutant to hydroxylamine. The slow growth of the parental strain and mutant strain when exposed to 500 μM hydroxylamine, a concentration 80-fold lower that the reported K_m of Hcp for hydroxylamine, suggests that even this low concentration is sufficient to greatly inhibit growth and places more doubt on the significance of Hcp-Hcr in hydroxylamine reduction. Initial investigations into the regulation of the *hcp* promoter suggested that transcription of *hcp* was activated by nitrite, via NarXL (Filenko *et al.*, 2005). However, more recent unpublished data has shown that the apparent up-regulation of *hcp* in response to nitrite is due solely to de-repression of NsrR and is independent of NarXL (Chismon, D unpublished data). In order to assess the effect of nitrite-dependent NsrR de-repression on hydroxylamine resistance a tandem experiment was designed to pre-adapt the strains to nitrosative stress by including nitrite in the medium. The nitrite present should induce expression of *hcp-hcr* by activating NarL and de-repressing NsrR, leading to an increased resistance to hydroxylamine in the parental strain, if Hcp contributes towards hydroxylamine resistance. In the parental strain a greater growth yield at 40 hours indicated that nitrite adaptation did lead to an increased resistance to hydroxylamine stress. However, nitrite adaptation also led to an increase in hydroxylamine resistance of the Hcp mutant, indicating that there are other systems that are up-regulated by nitrite adaptation that enable *E. coli* to resist the toxic effects of hydroxylamine.

The role of the Hcp in hydroxylamine resistance in sub-cultured bacteria

Simple test-tube growth yield experiments, detailed above, showed that there was a long lag in the recovery of both the parental strain and the *hcp* mutant when grown in the presence of hydroxylamine. Small differences in the growth yield of the two strains at 40-hours post inoculation suggested that there might be a slight increase in the sensitivity of the *hcp*

mutant to hydroxylamine. To investigate this effect further, an experiment was designed to determine the effect of a sub-lethal concentration of hydroxylamine on the parental and *hcp* null strains when growing at comparable rates (fig. 3.4). Strains were first grown aerobically in rich medium and then sub-cultured into minimal medium, without shaking, to adapt the bacteria to anaerobic growth before applying hydroxylamine stress. This culture was grown for approximately 12 hours, then used to inoculate a large volume of medium for each strain. This culture was grown to mid-exponential point before being split into three equal volumes, two of which were treated with 500 μM hydroxylamine and one was left untreated. This experimental design led to a resolution of the temporal difference in recovery from growth inhibition by hydroxylamine between the parental strain and mutant. In the parental strain, growth was inhibited for 5 hours post-treatment with hydroxylamine, but after this time the growth rate quickly recovered and the eventual yield after 8 hours was approximately equal to the untreated culture, indicating that the culture had become nutrient limited. In contrast, the *hcp* null mutant cultures only began to recover 8 hours after hydroxylamine addition. This result suggests that Hcp does confer some resistance to hydroxylamine stress, but that there is some other system present in *E. coli* that eventually allows the Hcp mutant to recover. The phenotype of the *hcp* null mutant during hydroxylamine stress was slight but highly reproducible, suggesting there was a real difference in the ability of the mutant to deal with the toxic effects of hydroxylamine, or another related toxic product.

The activity of the NADH dependent nitrite reductase, NirB, in an Hcp mutant during hydroxylamine stress

The regulation of *hcp-hcr* in response to nitrite and nitric oxide, via NsrR, suggests that Hcp expression is concomitant with the expression of the NADH-dependent nitrite reductase, Nir (Constantinidou *et al.*, 2006). As it has been shown previously that Nir can reduce hydroxylamine to ammonia and has a much lower K_m for hydroxylamine at pH 7 than Hcp (Jackson *et al.*, 1981; Wolfe *et al.*, 2002). Hcp expression would be redundant if the two

Fig. 3.4

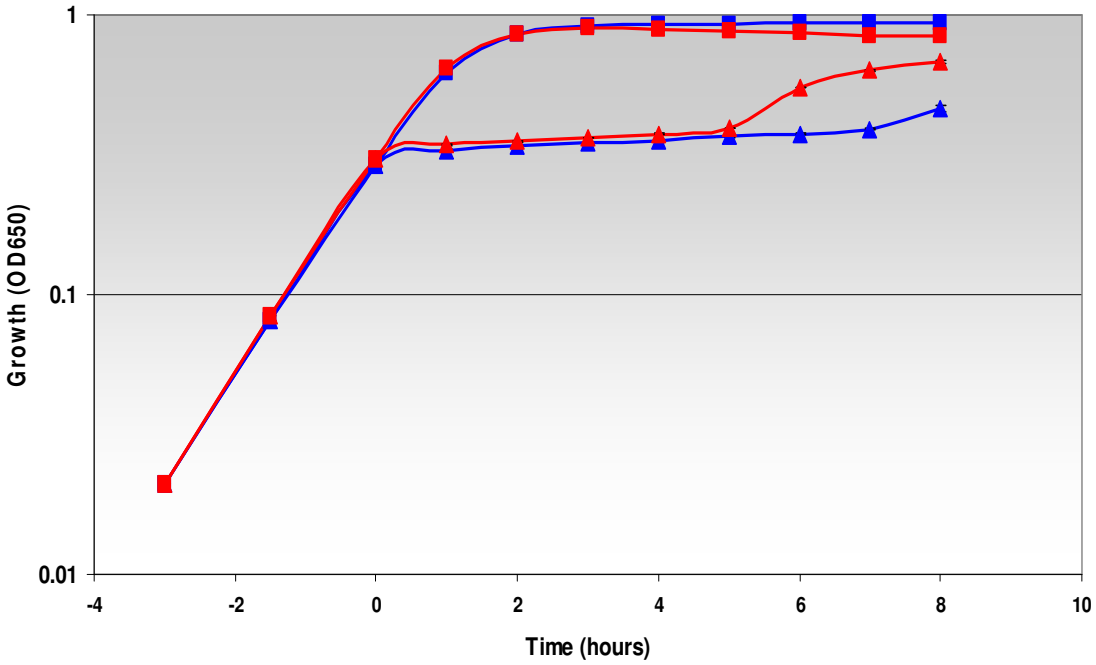

Fig. 3.4 Anaerobic growth inhibition of RK4353 and a *hcp* mutant strain, JCB5000, by hydroxylamine after sub-culture

Strains RK4353 (red) and JCB5000 (blue) were aerated at 37℃ in 5 ml of Lennox broth for 8 hours. This culture was then used to inoculate 100 ml of MS supplemented with 5% LB, 0.4% glycerol, 20 mM TMAO and 20 mM fumarate in a 100 ml conical flask. These cultures were then incubated at 30℃ without shaking for ~12 hours. A 10% inoculum of the overnight cultures was used to inoculate 500 ml of MS 5% LB supplemented with 0.4% glycerol, 20 mM TMAO and 20 mM fumarate in a 250 ml conical flask that was incubated at 37℃ without shaking until an optical density of ~0.3 was obtained. At this point (time point 0) the large cultures were split into three sterile, pre-warmed 100 ml conical flasks per strain, two containing 500 µM hydroxylamine (filled triangles) and the other containing an equal volume of sterile water (filled squares). Growth of all cultures was monitored for 8 hours post-shock. Error bars represent one standard deviation of the mean of the two treated cultures per strain

proteins were co-ordinately expressed. However, Hcp might fulfil a role in modulating NirB activity to direct it to reduce hydroxylamine if this toxic intermediate accumulates, or alternatively repair damage to NirB caused by nitrosative stress. In order to measure the activity of NirB in the parental strain and *hcp* mutant before, during and after hydroxylamine stress, a large volume of culture for each strain in each condition was harvested and the bacteria were broken in the French press. The cytoplasmic fraction, which contained the soluble nitrite reductase, NirB, was separated by ultra-centrifugation and was assayed for NirB-dependent nitrite reduction, by measuring the nitrite-dependent oxidation of NADH by spectrophotometry at 340 nm (fig. 3.5). No significant decrease in either the parental strain or the mutant strain was observed after treatment with hydroxylamine, indicating not only that NirB activity is unaffected by the presence of hydroxylamine, but also that Hcp does not modulate the function of NirB.

The effect of a *yeaR-yoaG* mutation on anaerobic growth in the presence of nitric oxide

As will be discussed in the next chapter, the *yeaR-yoaG* operon is maximally expressed in the presence of nitrate in the absence of functional FNR. The ability of FNR to function is governed by a coordinated iron-sulphur centre that is reduced to a [4Fe-4S] centre in the absence of oxygen allowing for protein dimerisation and DNA binding. It will be demonstrated in chapter 5 of this study, that the effect of functional FNR on the *yeaR-yoaG* promoter is indirect. However, the effect on expression is still dramatic, suggesting that it is under conditions when FNR function is negated that YeaR-YoaG is synthesized. Previous studies have shown that FNR is not only sensitive to oxygen, but is also deactivated by nitric oxide, which binds to the iron atoms of the iron-sulphur centre to cause the formation of S-nitroso adducts (Cruz-Ramos *et al.*, 2002). This evidence, taken with the fact that transcription of *yeaR-yoaG* is regulated by the nitric oxide sensitive repressor, NsrR, suggests that YeaR-YoaG may be required when nitrate reduction has led to overproduction of nitric oxide and caused

Fig. 3.5

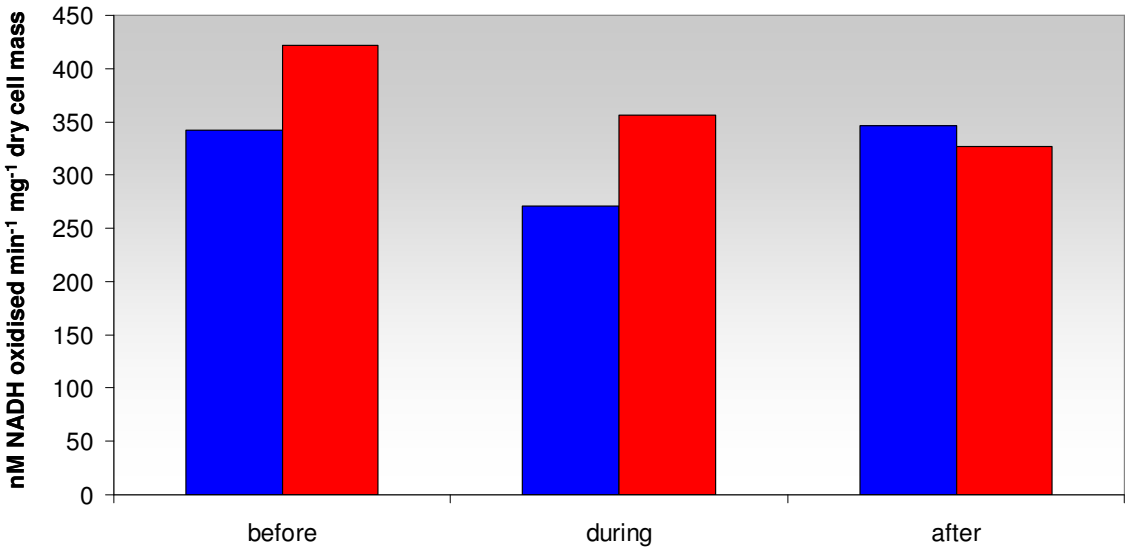

Fig. 3.5 The effect of HCP on NirB dependent nitrite reductase activity before, during and after hydroxylamine stress

Strains RK4353 (red) and JCB5000 (blue) were aerated at 37°C in 20 ml Lennox broth for 8 hours. This culture was then used to inoculate three flask per strain consisting of 1000 ml of LB supplemented with 0.8% glucose and 2.5 mM nitrite in a 1000 ml conical flask. Cultures were incubated at 37°C without shaking until an optical density of ~0.3 was obtained. At this point a the bacteria from one flask of each strain was harvested (before) and 200 μM hydroxylamine was added to the remaining cultures. After 2 hours of growth in the presence of hydroxylamine, a further flask of each strain was harvested (during) and finally after 8 hours of growth the remaining cultures were harvested (after). All cells were then lysed by french press, cytoplasm preps were obtained by ultra-centrifugation and activity of NirB in the cytoplasmic extracts was determined by measuring NADH oxidation via spectroscopic absorption at 540 nM.

damage to FNR.

In order to assess the effect of YeaR-YoaG on anaerobic growth in the presence of nitric oxide, a chromosomal deletion was constructed in which the coding region of *yeaR-yoaG* was replaced with a chloramphenicol resistance cassette using the lambda *red* system (Datsenko and Wanner, 2000). This mutant, designated JCB5100 was grown, in tandem with the parental strain, RK4353, overnight in 5 ml of LB. These cultures were used to inoculate three 100 ml conical flasks per strain, containing 100 ml of MS supplemented with 5% LB, 0.4% glycerol, 20 mM TMAO, and 20 mM fumarate (fig. 3.6). The cultures were grown at 37°C without shaking until mid-exponential growth stage (OD_{650} ~0.3) was reached, at which point two flasks per culture were treated with 100 μM NO and the third flask was treated with sterile water only (time point 0). The growth of the cultures was monitored for 5 hours post-stress and the effect on growth of the strains was observed. Both strains grew at very similar rates in the absence of any nitric oxide, indicating that YeaR-YoaG is not required for normal growth under these conditions. After addition of nitric oxide, the growth of both strains was equally inhibited for the same period of time and normal growth resumed in both strains after 1 hour post-shock. This suggests that YeaR-YoaG does not influence the growth rate in the presence of NO under the conditions tested.

The effect of a *yeaR-yoaG* mutation on anaerobic growth in the presence of hydroxylamine

As has already been discussed, hydroxylamine is produced when *E. coli* respires nitrite and it is also possible that hydroxylamine is produced as a secondary product of nitric oxide toxicity. As *yeaR-yoaG* expression is maximal during growth in the presence of nitrate, when endogenous nitric oxide and hydroxylamine are likely to be produced, the effect of hydroxylamine on the growth of a *yeaR-yoaG* null mutant was determined (fig. 3.7). The *yeaR-yoaG* mutant and the parental strain, RK4353, were grown overnight in LB. A 5% inoculum of this culture was used to inoculate three 100 ml conical flasks per strain containing 100 ml of

Fig. 3.6

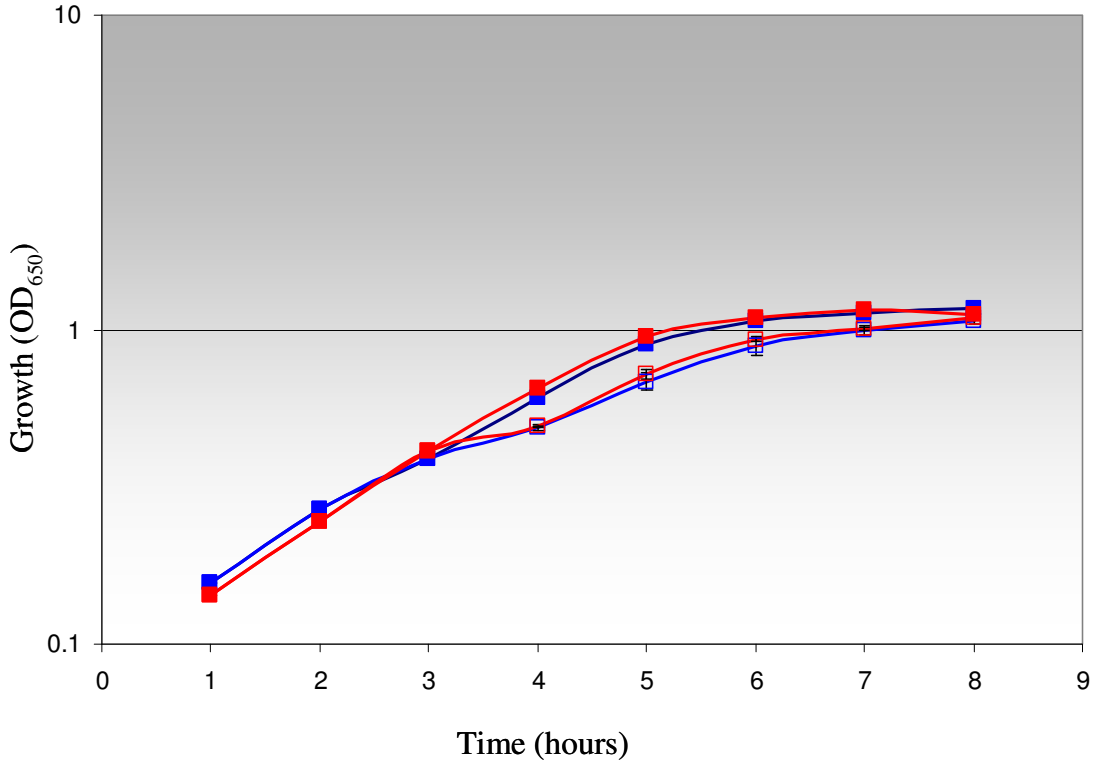

Fig. 3.6 The effect of YeaR-YoaG on anaerobic growth during nitric oxide stress

Overnight cultures of the parental strain, RK4353 (red), and a *yeaR-yoaG* mutant, RK4353Δ*yeaR-yoaG* (blue), were used to inoculate three 100 ml conical flasks per culture, containing 100 ml of MS medium supplemented with 5% LB, 0.4% glycerol, 20 mM TMAO and 20 mM fumarate. Cultures were incubated without shaking at 37°C until they reached mid-exponential growth phase (OD650 ~0.3) at which point a single pulse of 100 µM nitric oxide was introduced to two flasks per strain (open squares), without exposing the NOSW to the air (time point 0) while the remaining culture was treated with sterile water only (filled squares). The *y*-axis error bars represent one standard deviation of the mean of the two treated flasks per strain.

Fig. 3.7

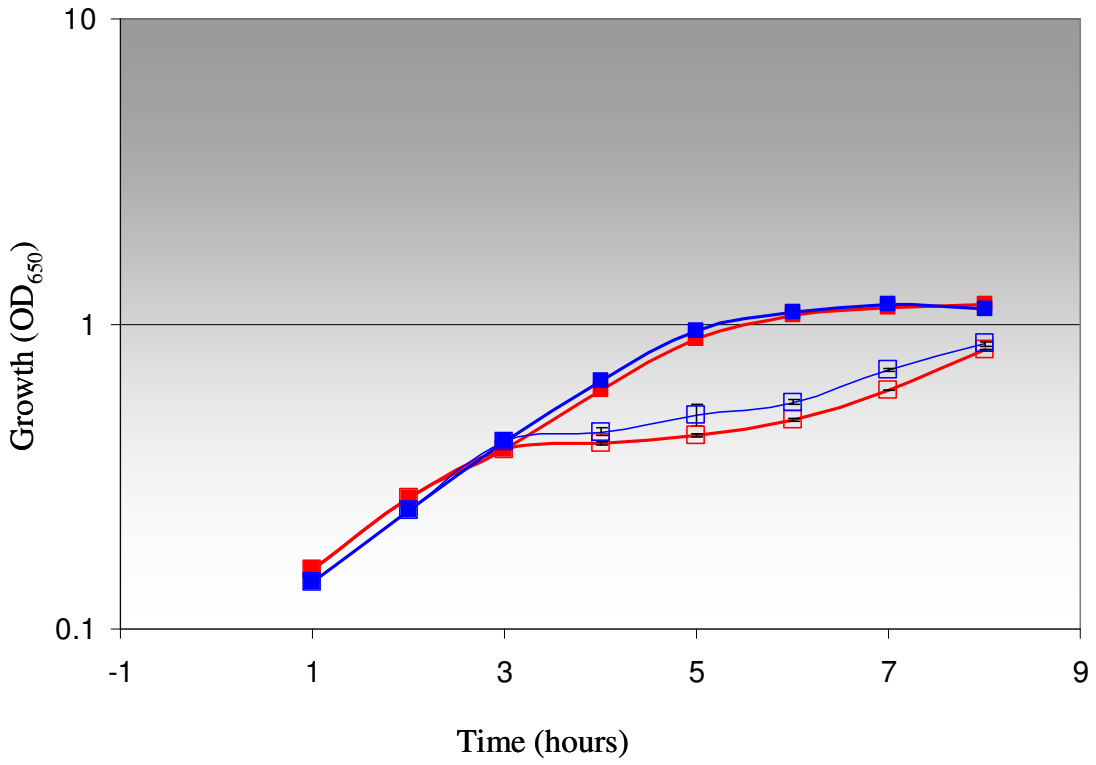

Fig. 3.7 The effect of YeaR-YoaG on anaerobic growth during hydroxylamine stress

Overnight cultures of the parental strain, RK4353 (red), and a *yeaR-yoaG* mutant, RK4353Δ*yeaR-yoaG* (blue), were used to inoculate three 100 ml conical flasks per culture, containing 100 ml of MS 5% LB medium supplemented with 0.4% glycerol, 20 mM TMAO and 20 mM fumarate. Cultures were incubated without shaking at 37°C until they reached mid-exponential growth phase (OD650 ~0.3) at which point 100 μM of hydroxylamine was introduced (time point 0) to two flasks per strain (open squares) while the remaining culture was treated with sterile water only (filled squares). The error bars represent one standard deviation of the mean of the two treated cultures per strain.

MS supplemented with 5% LB, 0.4% glycerol, 20 mM TMAO and 20 mM fumarate. All cultures were grown at 37°C without shaking until an OD_{650} of ~0.3 was reached. At this point, two flasks per strain were treated with 100 μM hydroxylamine and the third flask per strain was treated with an equal volume of sterile water only. The growth of all cultures was monitored for a further 5 hours. In both the parental strain and the mutant, a transient inhibition of growth was observed when hydroxylamine was introduced. The growth rate of both the parental strain and the mutant was entirely inhibited for 1 hour post-treatment, before recovering to a normal growth rate after 3 hours. After 5 hours, the growth yield of both the parental strain and mutant treated cultures had reached that of the untreated cultures, which had entered stationary phase after 3 hours due to nutrient limitation. This result suggests that YeaR and YoaG are not required for growth in the presence of hydroxylamine.

Discussion

From these experiments, it is still not clear what the function of either Hcp-Hcr or YeaR-YoaG might be, but it does appear that neither operon is absolutely necessary for anaerobic growth in the presence of nitric oxide or hydroxylamine. It is clear that the most likely condition where these proteins will function is when their coding genes are maximally expressed. Micro-array studies have implicated both Hcp-Hcr and YeaR-YoaG in dealing with nitrosative stress, due to their increased expression in the presence of nitrate and nitrite and their apparent regulation by the nitric oxide responsive regulator, NsrR (Constantinidou *et al.*, 2006; Filenko *et al.*, 2007). However, only a minor phenotype has been observed following deletion of *hcp-hcr* and no growth phenotype after delection of *yeaR-yoaG*. It is therefore prudent to try and fully understand the regulation of these gene products to attempt to ascertain the specific conditions when maximal expression occurs.

The regulation of p*hcp* has been studied in detail and is known to be FNR-dependent, and enhanced by the presence of nitrite via de-repression of NsrR (Filenko *et al.*, 2007; Filenko

et al., 2005). This fact certainly indicates that Hcp-Hcr is involved in anaerobic nitrate metabolism in some capacity. In contrast, expression of *yeaR-yoaG* appears to be FNR independent, activated in response to nitrate and nitrite and is repressed by NsrR (Filenko *et al.*, 2007). Other than this, very little is known about the regulation of *yeaR-yoaG* from sources other that microarray analysis and thus it is essential, if the function of YeaR-YoaG is to be identified, that the regulatory mechanisms that control the *yeaR* promoter are determined.

Chapter 4

Results

The regulation of the genes of unknown function, **yeaR-yoaG** *and comparative studies of the* **ogt** *promoter*

Introduction

The enteric bacterium, *Escherichia coli*, is able to respire both aerobically and anaerobically utilizing an array of terminal electron acceptors, including nitrate and nitrite (Cole, 1996). During the switch from aerobic to anaerobic growth, *E. coli* must synthesize the specific enzymes necessary to utilize any electron acceptors available in their environment. The synthesis of these enzymes is controlled mainly at the transcription level by a hierarchical system of transcription regulators (Goh *et al.*, 2005), which sense the chemical state of the environment and bring about a response to utilize the most powerful oxidant first (Stewart, 1993). Transcription is often controlled by a complex network of protein factors that respond to external signals and regulate promoter activity. Often, several factors are needed to bind together at a promoter in order to activate transcription (Salgado *et al.*, 2006). The major regulator of anaerobiosis is the global transcription regulator, FNR, which senses the absence of oxygen via a coordinated iron-sulphur centre (Khoroshilova *et al.*, 1997; Kiley and Beinert, 2003). When active, FNR is able to bind to DNA and act as either a transcription activator or a repressor (Constantinidou *et al.*, 2006). A further level of control is provided by the dual acting two-component regulators, NarXL and NarQP. The inner membrane-bound sensor kinases, NarX and NarQ, autophosphorylate in response to the presence of nitrate and nitrite in the periplasm and, in turn, phosphorylate the cognate response regulators, NarL and NarP. When phosphorylated, NarL and NarP bind DNA as either transcription activators or as repressors (Stewart, 1993; Stewart and Bledsoe, 2003)

In many cases, the transcription of genes for anaerobic respiratory enzymes requires the binding of both FNR and phospho-NarL/P for maximal expression (Stewart, 1993). The

expression of the nitrate reductase, *narGHI*, and the nitrite reductases, *nirB* and *nrfABCD*, require coordinate binding of both FNR and NarL to the promoter region. In the case of *narGHI*, FNR binds to the promoter region at a position centred between 41 and 42 bp upstream of the transcription start point, often defined as position -41.5. When bound in this position, FNR contacts the RNA polymerase holoenzyme and activates transcription in a class II manner (Schroder *et al.*, 1993). At the *narGHI* promoter, the binding of phospho-NarL to DNA in the upstream region of the promoter increases expression to maximal levels, but it is not thought that NarL contacts the RNAP directly (Stewart, 1993). At the promoter of *nirBDC*, encoding the NADH-dependent nitrite reductase (Peakman *et al.*, 1990), FNR and NarL also act co-ordinately (Jayaraman *et al*., 1987; Wu *et al*., 1998), In this case FNR binds at position -41.5, but in order for FNR-dependent transcription to proceed, NarL is required to overcome complex nucleoid folding, caused by IHF, by binding competitively at a site positioned at -61.6 (Browning *et al.*, 2004b). Transcription activation occurs by a similar mechanism at the *nrfABCD* promoter (Browning *et al*., 2005). These and several other promoters require a concomitant binding of FNR and NarL for maximal expression. At the start of this project there were no examples of NarL dependent activation independent of FNR that had been confirmed by extensive study. However, a number of candidate genes were identified by transcriptomic analysis (Constantinidou *et al.*, 2006).

In a reassessment of the extent of the FNR regulon, a group of genes were identified as being upregulated by NarL in response to nitrate, even in an FNR null strain *(Constantinidou et al*., 2006). Of these genes, the most dramatically activated by nitrate in a FNR null strain was the operon of unknown function, *yeaR-yoaG*. The relative expression levels of *yeaR-yoaG* in wild type and an FNR null strain would even suggest a possible FNR repression effect (Constantinidou *et al.*, 2006). A simultaneous transcriptomic study showed that *yeaR-yoaG* was one of a small group of genes shown to be upregulated by nitric oxide (Justino *et al.*, 2005b). Nitric oxide is generated endogenously as a by-product of nitrite reduction by both the

cytochrome-c nitrite reductase, NrfA, and the NADH–dependent nitrite reductase, NirB (Corker and Poole, 2003; Weiss, 2006) as well as by the innate immune response of infected hosts (Fang, 2004). Recently, the gene product of *yjeB*, NsrR, has been identified as a nitric oxide responsive transcription regulator (Bodenmiller and Spiro, 2006; Rodionov *et al*., 2005) and has subsequently been shown to control transcription of a small array of genes including *yeaR-yoaG*, where NsrR is thought to act as a repressor (Filenko *et al*., 2007). The transcriptomic study also predicted the putative NsrR site in the intergenic region between *yeaR-yoaG* and the upstream gene *leuE* (*yeaS*). However, as the promoter region was uncharacterized, it was not known whether the NsrR site overlaps the promoter region, as it has been shown to do at other promoters (Bodenmiller and Spiro, 2006; Filenko *et al*., 2007). A second *E. coli* promoter that was identified as a possible candidate for NarL dependent activation, independent of FNR, from transcriptomic studies, was the *ogt* promoter (Constantinidou *et al*., 2006). Again, this promoter was shown to be upregulated by the presence of nitrate, even in a strain in which FNR was not functional. This was highly significant as previous studies have shown that *ogt* encodes for the O6-alkylguanine DNA alkyltransferase, a protein that removes alkyl groups from chemically damaged guanine residues (Margison *et al*., 1985; Potter *et al*., 1987). This implicated *ogt* in repairing damage caused to DNA by nitrosative stress generated while the bacterium is respiring nitrate. During this project, a tandem study by Meng Xu (PhD thesis, University of Birmingham) demonstrated that induction of the *ogt* promoter by nitrate ions is NarL-dependent. and that NarL bound directly to the promoter region in at least one site. It was also demonstrated that Fis binds to the promoter region (Meng Xu, thesis. University of Birmingham). However, characterisation of the promoter was not fully completed at the end of this study so the *ogt* promoter was used as a comparative promoter for study of NarL dependent activation in this project.

During this project an independent study confirmed the findings of the FNR regulon investigation, by *in vivo* promoter investigation, that the operon *yeaR-yoaG* is regulated in a NarL-dependent, FNR-independent manner (Lin *et al.*, 2007). In most results, the study by Lin *et al*., (2007) was comparible to this study and, where different, was discussed. However, this study aimed to further characterise the *yeaR* promoter and to define the interactions of the relevant transcription factors with the promoter DNA.

Determination of the *yeaR-yoaG* transcription start site

In order to facilitate the analysis of the *yeaR* promoter region, it was necessary to map the position of the transcription start point (+1). The method of reverse transcription primer extension was chosen to identify the transcription start point. The strain, JCB387, was transformed with the *yeaR100* promoter fragment, cloned as a promoter-*lacZ* fusion in pRW50, and grown anaerobically in the presence of 20 mM nitrate, conditions optimal for *yeaR-yoaG* expression. At mid-exponential growth phase, the cells were harvested and total RNA was extracted. The *yeaR-yoaG* mRNA was used as a template for the synthesis of cDNA using reverse transcriptase and an oligonucleotide primer, specific for a region of the pRW50 vector sequence 83-nt downstream of the vector *Hin*dIII site. The cDNA product was separated by PAGE alongside a di-deoxy termination sequence ladder, generated by sequencing plasmid pSR using primer D36245, for size comparison (Fig. 4.1). Primer extension revealed a single, abundant transcript. The size of this transcript was mapped onto the sequence of the cloned promoter fragment (Fig 4.2). The calculated transcription start site, nucleotide G, is labelled +1. A putative σ^{70}-RNA polymerase -10 promoter element, which showed 3 out of 6 nt sequence similarity to the consensus sequence (TATAAT) was identified and annotated on the sequence. The sequence of the putative -35 promoter element also showed 4 out of 6 nt similarity to the consensus sequence (TTGACA). The -10 and -35 promoter elements are

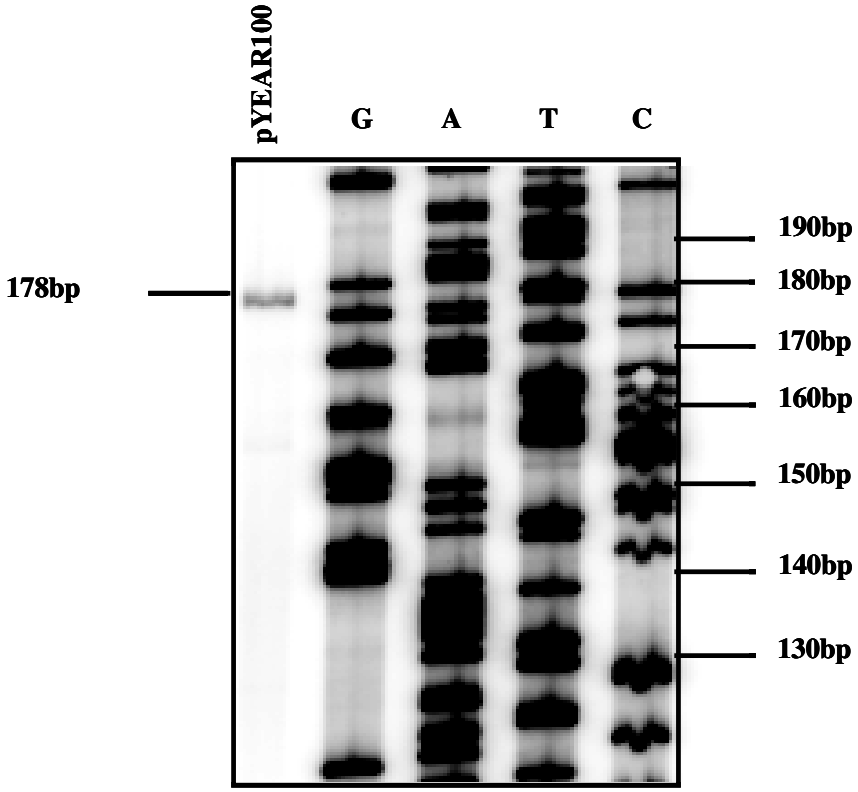

Figure 4.1 Transcription start site mapping using primer extension

E. coli strain JCB387 was transformed with pRW50 plasmid, carrying the *yeaR100* promoter fragment, and grown anaerobically at 37°C, without stirring, in 100 ml of minimal salts medium supplemented with 5% LB, 0.4% glycerol and 20 mM $NaNO_3$ in a 100 ml conical flask. At the mid-exponential phase growth, OD_{650} ~0.7, bacteria were harvested and total cellular mRNA was extracted. The cellular mRNA was then used as a template for cDNA synthesis using reverse transcriptase and a radio-labeled oligonucleotide (D49724), which binds specifically to a region of the pRW50 vector 85-nt downstream from the *Hin*dIII site. The cDNA product was then separated by polyacrylamide electrophoresis and its size was determined by comparison with a di-deoxy sequence ladder generated from pSR vector DNA. The size of the fragment was shown to be 178 nt, which places the transcription start site 95 nt upstream of the first base of the *Hin*dIII site.

spaced 17 nt apart, a distance optimal for promoter activiy. This transcription start site was also identified by RACE by Lin *et al.,* (2007).

In silico identification of putative promoter elements

Once the transcription start site had been identified, and the promoter elements mapped, the inverted repeat of the NsrR binding site (Filenko *et al*., 2007) was located on the promoter sequence. As with other promoters repressed by NsrR (Filenko *et al*., 2007), the NsrR binding sequence overlapped the putative -10 and -35 promoter elements (Fig 4.2). Further sequence analysis, using the online bioinfromatic tools, FUZZNUC and SRS, revealed several putative NarL heptamers, which had a 5 out of 7 or better match with the consensus sequence TACYNMT. A single 7-2-7 inverted repeat sequence, the optimal binding configuration for NarL and NarP, with acceptable sequence similarity (3 mismatches) to the consensus sequence was also found at a position -43.5 nt upstream with respect to the transcription start site (Fig 4.2). Two further putative NarL sites were identified downstream of the translation start site of *yeaR*, within the coding region. In addition, several putative binding sites for the nucleoid associated protein, Fis, were identified in the promoter region, overlapping the putative NarL site.

Phospho-NarL is required for transcription activation at the *yeaR* promoter

To analyse the dependence of p*yeaR* activity on functional NarL/ P, the *yeaR* promoter fragment, *yeaR100*, was cloned as an *Eco*RI-*Hin*dIII fragment into the low copy *lacZ*-fusion vector, pRW50. The resultant plasmid, pYEAR100, was transformed into a parental *E. coli* strain, JCB387, and NarP, NarL and NarLP null derivatives: JCB3875, JCB3883 and JCB3884, respectively. In this experiment, and all subsequent β-galactosidase activity assays, all transformants were grown anaerobically in minimal medium supplemented with 5% LB, glycerol (0.4%), fumarate (20 mM), TMAO (20 mM) and in the presence or absence of either nitrate (20 mM) or nitrite (2.5 mM) unless otherwise stated. When cultures reached the mid-

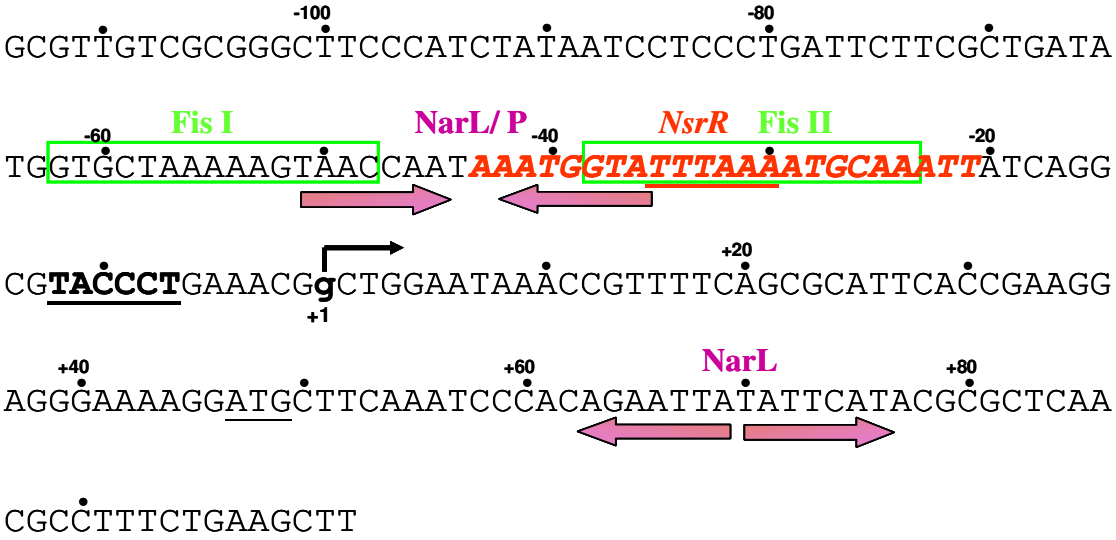

Figure 4.2 Bio-informatic determination of the promoter structure

Using the experimentally determined transcription start site (lower case **g),** the predicted -10 and -35 promoter elements were identified (**bold underlined sequence**). The *yeaR-yoaG* promoter sequence was checked for putative transcription factor binding sites using online bio-informatic tools, fuzznuc and SRS. A number of putative sites were identified in the promoter region, including NarL (purple arrows) NarL/ P (purple arrows in 7-2-7 inverted repeat configuration), Fis (green boxed sequence) and NsrR (red, italic, bold text).

exponential phase of growth, cells were harvested, lysed and assayed for β-galactosidase activity (table 4.1). In the parental strain, maximal promoter activity was observed in the presence of nitrate. Transcription induction was also observed in the parental strain in the presence of nitrite, but to a lesser extent. In the NarP null strain, JCB3875, activation by nitrate was only slightly diminished, suggesting that NarP has, at most, a limited role at this promoter. The effect of a NarP mutation in the presence of nitrite also reflects this. Conversely, a total loss of transcription activation in the presence of either nitrate or nitrite was observed in the NarL mutant suggesting an absolute requirement for functional NarL for transcription activation at the *yeaR* promoter. This conclusion is further supported by the activity in the NarLP double mutant. These data suggest that NarL is absolutely required for maximal activity from the *yeaR* promoter in response to the presence of nitrate. Similar results were resported by Lin *et al*., (2007)

Phospho-NarL binds directly to the *yeaR* promoter

In order to ascertain whether the dependence of *yeaR* on NarL is due to direct binding of NarL to the promoter region, the *yeaR100* promoter fragment was purified and end-labelled using γP^{32}dATP for use in an electromobility shift assay. The promoter fragment was incubated with purified NarL that had been phosphorylated with acetyl phosphate. Subsequent DNA-protein complexes were separated by PAGE and visualized by phospho-imaging (Fig 4.3). Two bandshifts can be observed as the concentration of phospho-NarL was increased, suggesting that there are two binding sites for NarL, one high affinity site and one low affinity site.

The high affinity binding site for NarL is located within 148 bp upstream of the *yeaR* transcription start site

The full length *yeaR100* promoter fragment not only contained the intergenic region between *yeaR-yoaG* and the upstream gene *leuE* (*yeaS*), but also included ≈170 bp of the *leuE* open reading frame. To show whether this region, which also contained candidate NarL binding sites, binds NarL *in vitro,* a second gel retardation assay was designed in which the full

Table 4.1 The activity of the *yeaR-yoaG* promoter is NarL dependent.
Expression from the *yeaR* promoter, cloned as a promoter-*lacZ* fusion in pRW50, was measured by β-galactosidase activity assay. All *E. coli* K-12 strains were transformed and grown anaerobically in minimal medium in the presence and absence of nitrate (20 mM) or nitrite (2.5 mM). All values represent the average of at least three independent assays and are expressed in units of nmol ONPG hydrolysed min^{-1} mg^{-1} dry cell mass. Error is represented as one standard deviation from the mean.

		Growth conditions		
Strain	Relevant mutations	Anaerobic	Anaerobic + NO_3^-	Anaerobic + NO_2^-
JCB387	*narL*$^+$ *narP*$^+$	96 ± 32	2204 ± 89	1032 ± 35
JCB3875	*narL*$^+$ *narP*	69 ± 0	1430 ± 196	239 ± 6
JCB3883	*narL narP*$^+$	166 ± 69	160 ± 108	113 ± 7
JCB3884	*narL narP*	75 ± 5	88 ± 3	102 ± 7

Fig. 4.3

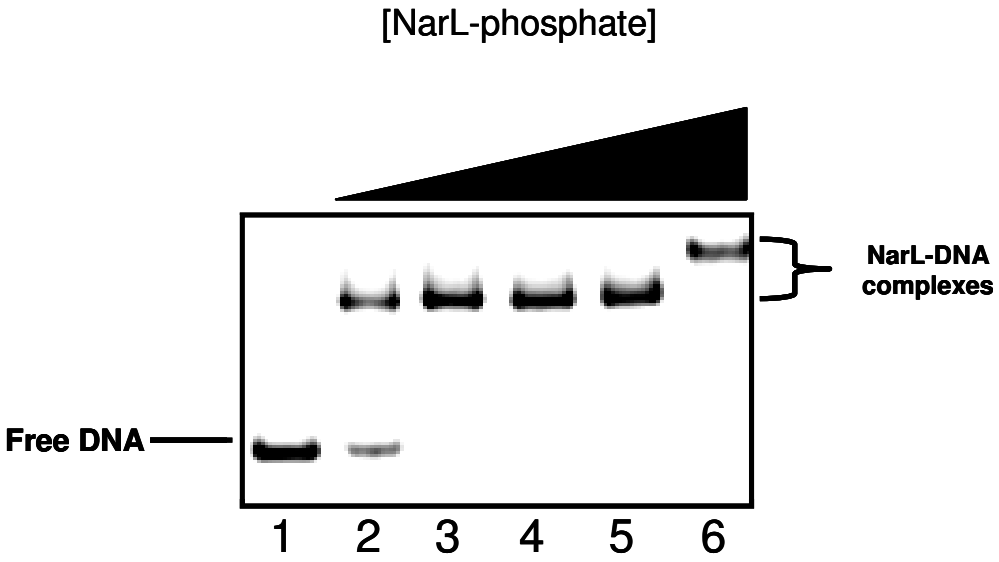

Fig. 4.3 Phospho-NarL binds to the *yeaR-yoaG* promoter

The *Eco*RI-*Hin*dIII, *yeaR-yoaG,* promoter fragment was incubated with increasing concentrations of NarL and protein-DNA complexes were separated by polyacrylamide electrophoresis. NarL was phosphorylated prior to mixing with *yeaR* promoter DNA by incubating with 500 μM acetyl-phosphate for 20 minutes at 37°C. Lane 1 contains *yeaR-yoaG* promoter DNA only while lanes 2-6 contain 0.2, 0.4, 0.8, 1.6 and 3.2 μM [acetyl phosphate-treated NarL] respectively.

length fragment was digested with *Bgl*I before being incubated with NarL (fig. 4.4). This enzyme cut the fragment at only one site, generating two fragments of different sizes. The smaller fragment, upstream of the *Bgl*I site, contained the majority of the *leuE* ORF and the larger fragment downstream of the *Bgl*I site contained the intergenic region. The digested fragments were incubated with NarL protein and the DNA-protein complexes were separated by PAGE. The larger of the *Bgl*I digested fragments showed a band shift at the same NarL concentration as the high affinity NarL shift in the full length fragment. This suggests that the high affinity NarL binding site is located in the intergenic region between *yeaR* and *leuE*, within 148 bp upstream of the *yeaR-yoaG* transcription start site.

NarL binds to the 7-2-7 binding site centred around -47.5 with respect to the *yeaR* transcription start site

DNase I footprinting was used to identify the *in vitro* binding site for phospho-NarL at the *yeaR* promoter (Fig. 4.5). The *yeaR100 Aat*I-*Hin*dIII promoter fragment was end labelled with γP^{32}dATP and incubated with increasing concentrations of phospho-NarL before being treated with DNase I, which digests unprotected DNA. Digested fragments were separated by PAGE and calibrated using Maxam-Gilbert 'G+A' sequencing reactions, generated from the radio-labelled *yeaR100* fragment. At 0.8 μM NarL, a zone of protection or footprint can be seen in the region of the promoter between positions -51 and -21 with respect to the transcription start site. As protein concentration increases to 1.6 and 3.2 μM a clear footprint can be seen in this region centred at position -43.5 with respect to the transcription start site, the same site identified in bioinformatic analysis of the promoter region (fig 4.2). At high concentrations of NarL, several hypersensitive sites can be seen where NarL may bind to other sites, changing the conformation of the DNA and inducing digestion. However, no secondary footprint was observed despite an apparent second electromobility shift with NarL (fig. 4.4). The positioning of NarL at a site centred at position -43.5 with respect to the transcription start site is similar to the positioning of FNR at class II activated promoters.

Fig. 4.4

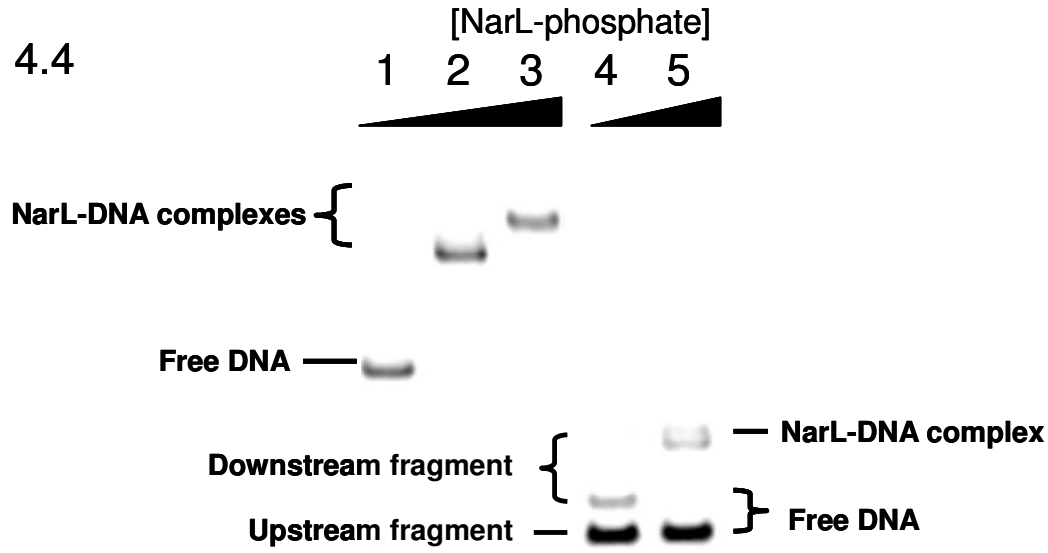

Fig. 4.4 The high affinity NarL binding site is located in the intergenic region between *yeaR-yoaG* and *leuS*

The *Eco*RI-*Hin*dIII, *yeaR-yoaG,* promoter fragment was end-labelled (1-3) then digested with *Bgl*I (4 and 5), which cut once at a position 148 nt upstream of the transcription start site to produce two fragments of different sizes. Both fragments were incubated with NarL, which had been phosphorylated with 500 μM acetyl phosphate, and DNA-protein complexes were separated by PAGE. Lanes 1 and 4 contain no protein. Lanes 2 and 5 contain 1.6 μM and lane 3 contains 3.2 μM NarL.

Figure 4.5

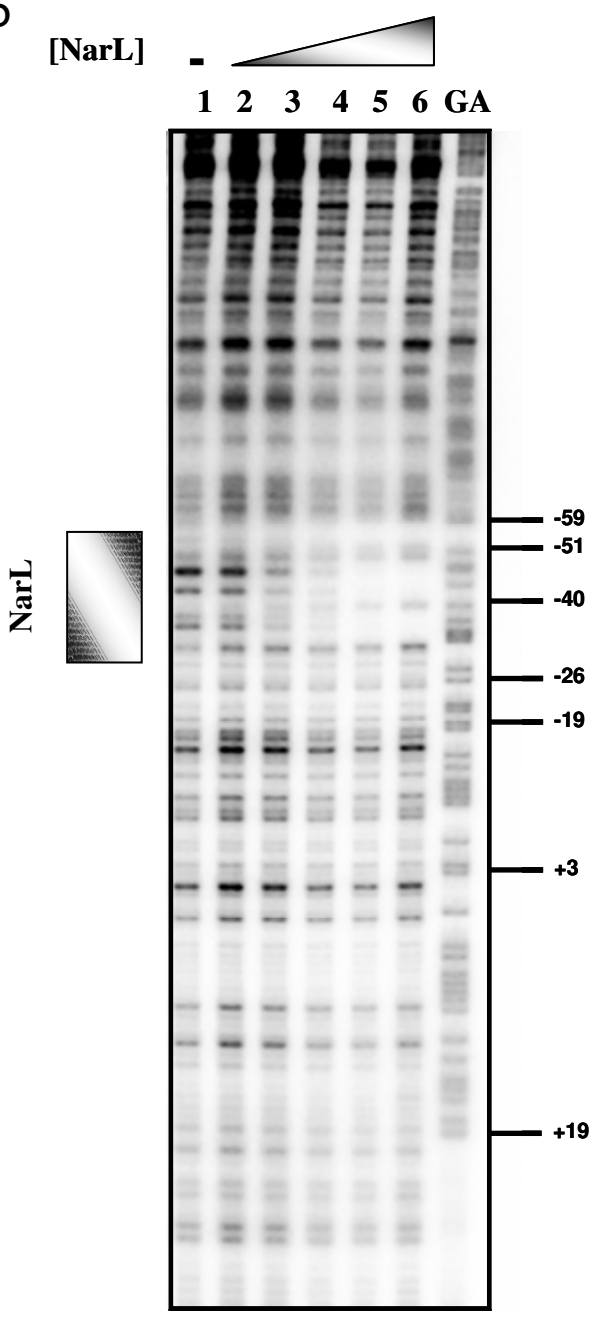

Figure 4.5. NarL binds to a single 7-2-7 site at the *yeaR* promoter.

An *in vitro* DNase I footprint experiment with purified NarL was used to identify the binding site of phospho-NarL at the *yeaR-yoaG* promoter. End-labelled *pyeaR Aat*II-*Hin*dIII fragment was incubated with increasing concentrations of NarL, which had been phosphorylated with acetyl phosphate prior to use, and subjected to DNase I digestion. The concentration of NarL was: lane 1, no protein; lane 2, 0.2 μM; lane 3, 0.4 μ M; lane 4, 0.8 μ M; lane 5, 1.6 μ M and lane 6, 3.2 μ M. DNA digest fragments were separated by PAGE and gels were calibrated using Maxam-Gilbert 'G+A' sequencing reactions. Relevant DNA positions are indicated. The location of the NarL binding site is shown as a shaded box.

Purified NarP does not bind to the *yeaR* promoter

When the activity of the *yeaR-lacZ* promoter fusion was assayed in a strain lacking functional NarP, a slight decrease in overall promoter activity was observed in comparison to the parental strain (table 4.1). This effect was less dramatic than that of a *narL* mutation, which caused a loss of all promoter activation. DNase footprint analysis revealed that the NarL operator in the *yeaR* promoter region was arranged as a 7-2-7 inverted repeat sequence, which can also be an operator for NarP (Darwin *et al.*, 1997). An EMSA was designed in order to ascertain whether purified phospho-NarP could bind to the *yeaR* promoter region *in vitro*. The *yeaR100* promoter fragment was end-labelled using γP^{32}-ATP and incubated with increasing concentrations of phosphorylated NarP protein. DNA-protein complexes were separated by PAGE and visualised by phospho-imaging (fig 4.6). No No bandshift was observed at any concentration of NarP tested, which suggests that NArP does not bind directly to the *yeaR* promoter, which is inconsistent with the results in table 4.1 and has not been explained.

In vivo transcription activation of p*yeaR* is repressed by FNR and NsrR

Due to the apparent repression of p*yeaR* by FNR and NsrR, revealed by micro-array analysis (Constantinidou *et al*., 2006; Filenko *et al*., 2007), the activity of the *yeaR100-lacZ* fusion in pRW50 was assayed in the presence and absence of functional FNR and NsrR (Figure 4.7). The *yeaR100-lacZ* promoter fusion was transformed into the parental strain, JCB387, and derivatives lacking in functional NsrR, FNR and both FNR and NsrR. The activity of the promoter during anaerobic growth, in the presence and absence of nitrate or nitrate, was determined using a β-galactosidase assay. In the absence of functional NsrR, activity of the *yeaR* promoter was 20% higher than the parental strain in the presence of nitrate and 2-fold higher in the absence of either nitrate or nitrite. However, the activity in the presence of nitrite was unchanged. Conversely, activity of p*yeaR* in the FNR null derivative

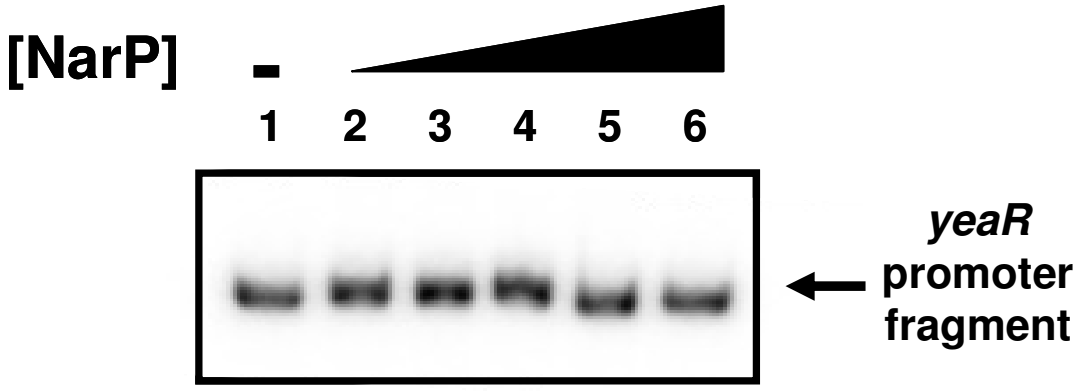

Fig. 4.6 Phospho-NarP does not bind to the *yeaR-yoaG* promoter

The *Eco*RI-*Hin*dIII, *yeaR-yoaG,* promoter fragment was incubated with increasing concentrations of purified NarP, and protein-DNA complexes were separated by polyacrylamide electrophoresis. Lane 1 contains *yeaR-yoaG* promoter DNA only while lanes 2-6 contain 0.4, 0.8, 1.6, 3.2 and 6.4 μM [NarP] respectively. NarP was phosphorylated prior to mixing with *yeaR* promoter DNA by incubating with 500 μM acetyl-phosphate for 20 minutes at 37°C.

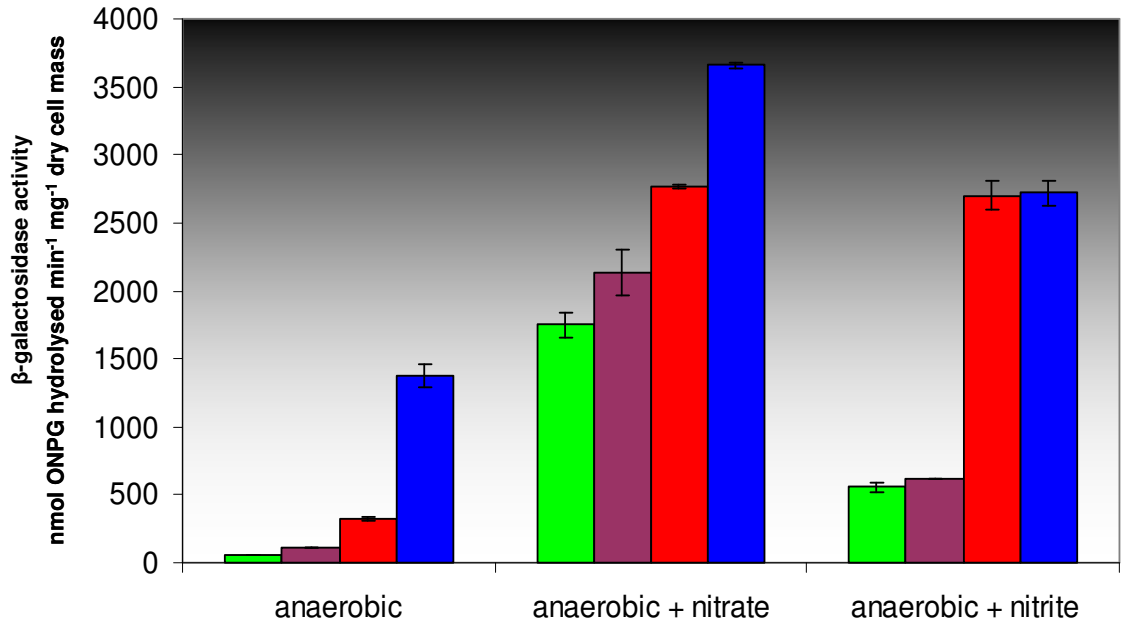

Figure 4.7 The effect of FNR and NsrR on transcription activation at the *yeaR-yoaG* promoter

Expression from the *yeaR100* promoter fragment, cloned as a promoter::*lacZ* fusion in pRW50 was measured by β-galactosidase activity assay in the parental strain JCB387 (green), JCB387Δ*nsrR* (burgundy), JCB387Δ*fnr* (red) and JCB387Δ*fnr*Δ*nsrR* (blue). At an OD650 of 0.6, cells were harvested, lysed and assayed for β-galactosidase activity. In this and all subsequent figures, β-galactosidase activity is expressed as nmol ONPG hydrolysed min^{-1} mg^{-1} dry cell mass. Data shown are means from at least 3 independent experiments with error bars shown as one standard deviation from the mean.

was 2-fold higher in the presence of nitrate and over 5-fold higher in the presence of nitrite, suggesting a strong effect of FNR function on the activity of p*yeaR*. In the *fnr nsrR* double mutant, the activity of the *yeaR* promoter was further increased in the presence of nitrate, but was unchanged in the presence of nitrite when compared to the FNR null mutant, suggesting that the effect of FNR in these conditions was more significant than that of NsrR. Interestingly, in the absence of nitrate or nitrite, the promoter was much more active in the *fnr nsrR* double mutant than in the parental strain. However, transcription was still induced by nitrate or nitrite. In the case of NsrR, a putative binding site in the *yeaR* promoter has been identified in this study and previously (Filenko *et al*., 2007). However, despite promoter activity being higher in the *fnr* mutant, no convincing putative FNR binding sites have been identified in the promoter region. The apparent repression of p*yeaR* by FNR could therefore be indirect and will be investigated in detail in chapter 5. These results were anaomalous to the findings of Lin *et al*., (2007) who did report that FNR and NsrR, but observed higher fold repression by NsrR.

The nucleoid-associated protein, Fis, represses transcription of *yeaR-yoaG in vivo*

In recent studies, using chromatin immuno-precipitation run on chip (ChIP-chip), the distribution of DNA-binding proteins across the entire chromosome has been investigated. An investigation of the distribution of nucleoid-associated protein binding sites using this method, identified a possible binding signal for Fis in the *yeaR-yoaG* promoter region (Grainger *et al*., 2006). The effect of Fis on the activity of p*yeaR* was therefore determined. The *yeaR100* promoter-*lacZ* fusion plasmid was transformed into the parental strain, JCB387, and the Fis null derivative, JCB3871, and the subsequent transformants were grown in both rich medium (LB) and minimal medium (MS), both in the presence and absence of nitrate. Results show that nitrate-induced activity from the *yeaR* promoter is severely restricted during growth in rich medium (Lennox broth) supplemented with glucose and that this suppression is relieved in the *Δfis* derivative (fig. 4.8). A similar trend of activity was observed for cultures grown in

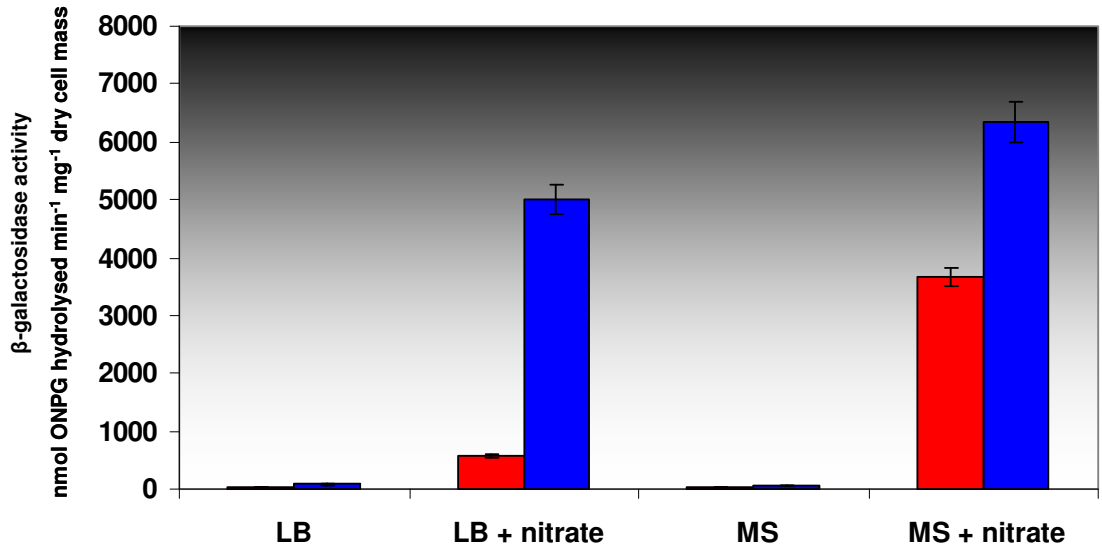

Fig. 4.8 The effect of Fis on transcription activation at the *yeaR-yoaG* promoter

Expression from the *yeaR100* promoter fragment, cloned as a promoter*::lacZ* fusion in pRW50, was measured by β-galactosidase assay in strains JCB387 (parental strain) (red) and JCB387Δ*fis* (blue) . Transformants were grown in in rich medium (Lennox broth) and minimal medium (MS) anaerobically in the presence or absence of nitrate (20 mM). At an OD_{650} of 0.6, cells were harvested, lysed and assayed for β-galactosidase activity.

minimal medium supplemented with glycerol. The strong repression effect of Fis on the activity of the *yeaR* promoter and the evidence from ChIP-chip analysis argue that Fis is a direct repressor of *yeaR-yoaG* expression.

NarL and Fis compete for a binding site in the *yeaR-yoaG* promoter region

The two possible binding sites for Fis, identified by bio-informatic searching (fig. 4.2), both overlapped the experimentally determined NarL binding site (fig. 4.6). It was therefore prudent to identify the binding site of Fis, *in vitro,* and to assess the effect of competitive binding between NarL and Fis at the *yeaR* promoter. The *yeaR100*, *Aat*I-*Hin*dIII fragment was end-labelled with γP^{32} dATP and incubated with increasing concentrations of Fis, in the presence and absence of NarL at a concentration shown previously to cause a footprint at the *yeaR* promoter. Protein-DNA complexes were then treated with DNase I and the resultant fragments were separated by PAGE and calibrated by comparison to a Maxam-Gilbert 'G+A' sequencing reaction, generated from the radio-labelled *yeaR100* fragment (fig. 4.9a). In the presence of Fis alone, two binding sites were observed and are indicated by shaded boxes. The first high affinity Fis site, Fis I, was visible after the addition of 0.22 μM Fis and was shown to be centred around -55 with respect to the transcription start site. At higher concentrations of Fis (0.45 μM) a second lower affinity site, Fis II, was observed, centred around -31 with respect to the transcription start site. A single hypersensitivity site caused by Fis binding was identified at position -29 (■). The location of the experimentally determined Fis binding sites matched the position of the bio-informatically predicted Fis sites (fig. 4.2). When the promoter region was incubated with increasing concentrations of Fis in the presence of a fixed concentration of NarL (lanes 6-10), a competition for overlapping binding sites for Fis and NarL was observed. In the absence of Fis, NarL causes a footprint centred around -43.5 with respect to the transcription start site and introduces two characteristic hypersensitivity sites at positions -50 and -40 with respect to the transcription start site (□). At high concentrations of Fis, (lanes 9

Figure 4.9

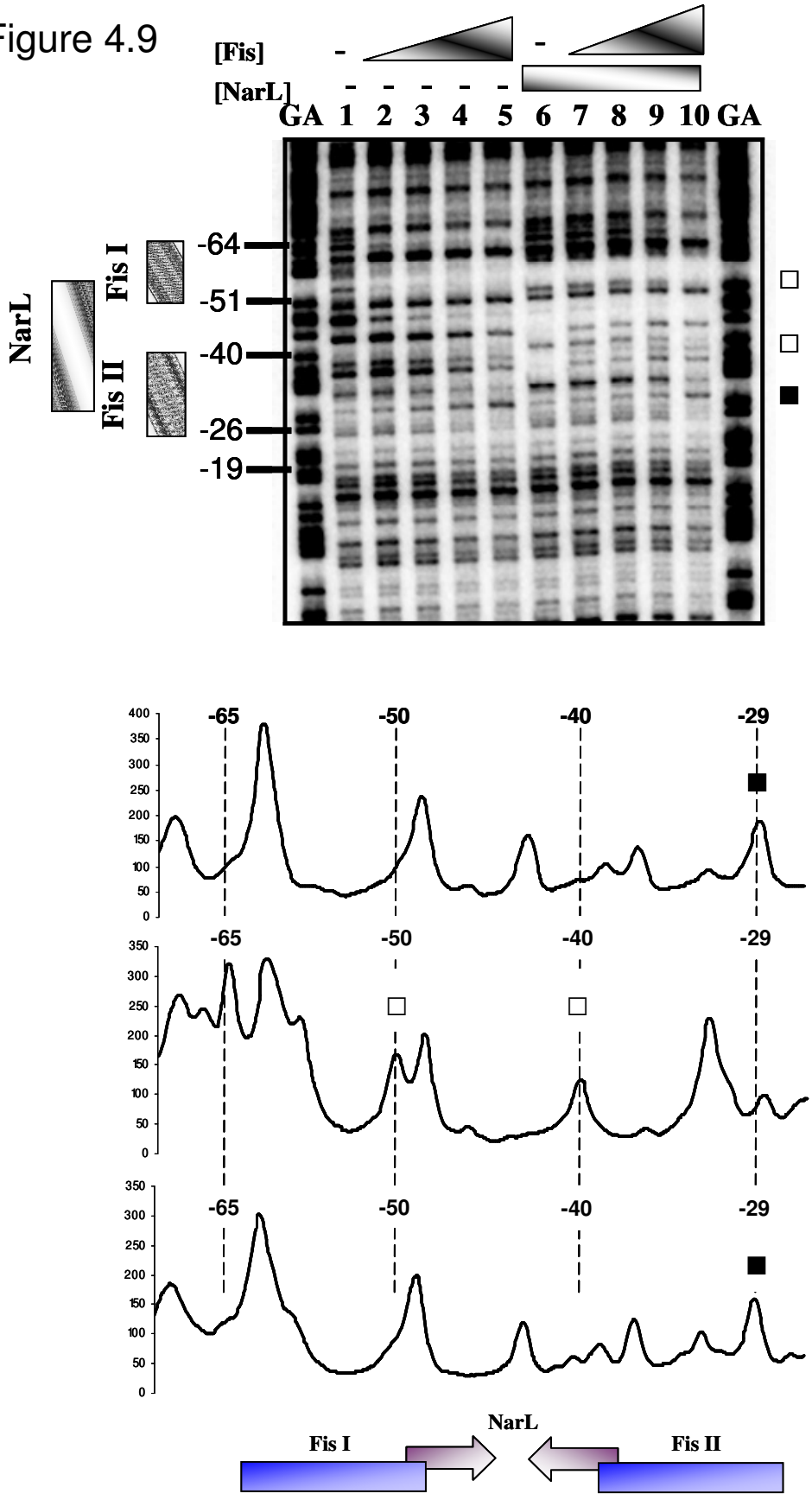
[Fis]
[NarL]
GA 1 2 3 4 5 6 7 8 9 10 GA
-64
-51
-40
-26
-19
NarL
Fis I
Fis II
-65
-50
-40
-29
Fis I
NarL
Fis II

Figure 4.9 NarL and Fis compete for binding at the *yeaR* promoter.

A. End-labelled *yeaR100 Aat*II-*Hin*dIII fragment was incubated with increasing concentrations of Fis in the presence and absence of a fixed concentration of NarL and subjected to DNase I footprint analysis. The concentration of Fis was lanes: 1 and 6, no protein; lanes 2 and 7, 0.22 mM; lanes 3 and 8, 0.45 mM; lanes 4 and 9, 0.90 mM; lanes 5 and 10, 1.8 mM. The concentration of NarL was lanes 1-5, no protein; lanes 6-10, 1.6 mM. Gels were calibrated using Maxam-Gilbert 'G+A' sequencing reactions and relevant positions are indicated. The location of the NarL and Fis binding sites are shown by boxes. Hypersensitive sites at positions -52 and -40, which are induced by NarL binding are indicated by the open boxes, whereas the hypersensitive site at position -29, which is induced by Fis binding, is marked by the filled boxes. Purified NarL was pre-treated with 500 μM acetyl phosphate before being used in these experiments.

B. Quantity-one software (BIORAD) was used to quantify Fis and NarL binding. A lane profile was generated for lane 5 (Fis only), lane 6 (NarL only) and lane 10 (NarL and Fis) based on the relative intensity of bands in the region containing both the Fis and NarL binding sites. This data was exported to Excel (microsoft) to generate histogram traces. Hypersensitive sites at positions -52 and -40, which are induced by NarL binding are indicated by the club symbol, whereas the hypersensitive site at position -29, which is induced by Fis binding, is marked by a spade symbol.

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and 10) a reduction in the binding of NarL was observed concomitantly with an increase in the binding of Fis, suggesting there is a dynamic competition for binding at the *yeaR* promoter. It was concluded that Fis represses the binding of the transcription activator, NarL, by steric hindrance. The apparent change from a NarL footprint to a Fis footprint with increasing concentrations of Fis was visualised by comparing the band intensities of lanes 5 (Fis only (i)), lane 6 (NarL only (ii)) and lane 10 (NarL + Fis (iii)), using the lane quantify tool in Quantity-one software (BioRad) (fig 4.9b). This information is displayed as a densitometry trace of relative intensity compared to distance travelled and clearly shows the appearance of Fis dependent hypersensitivity sites (■) and the loss of NarL dependent hypersensitivity sites (□) when both proteins are incubated with the *yeaR100* promoter fragment.

NarL enhances *yeaR* promoter opening *in vitro* while Fis represses

As NarL had been shown to activate *yeaR-yoaG* expression *in vivo*, the effect of NarL on promoter opening was investigated by potassium permanganate footprinting. The *Aat*I-*Hin*dIII *yeaR100* promoter fragment was end-labelled and incubated with a fixed concentration of RNAP σ70 holo-enzyme and increasing concentrations of NarL (fig. 4.10a). DNA-protein complexes were then treated with potassium permanganate, which attacks DNA in an open conformation and causes base modifications that are prone to cleavage. In the absence of NarL, RNAP facilitated promoter opening in a region between -2 and -11 with respect to the transcription start site (lane 1). This opening was increased in the presence of NarL, seen in lane 3 as a 1.4-fold increase in intensity of bands generated by DNA cleavage. This increase in open complex formation was more evident when the band intensities were quantified using Quantity-one software (BioRad) and displayed as a densitometry trace (fig 4.10b). An increase in intensity caused by the increase in NarL-dependent open-complex formation in lane 3 (red trace) could be seen clearly when compared to the intensity in the absence of NarL in lane 1 (green trace). This suggested that NarL directly activate transcription at the *yeaR* promoter.

Fig 4.10a

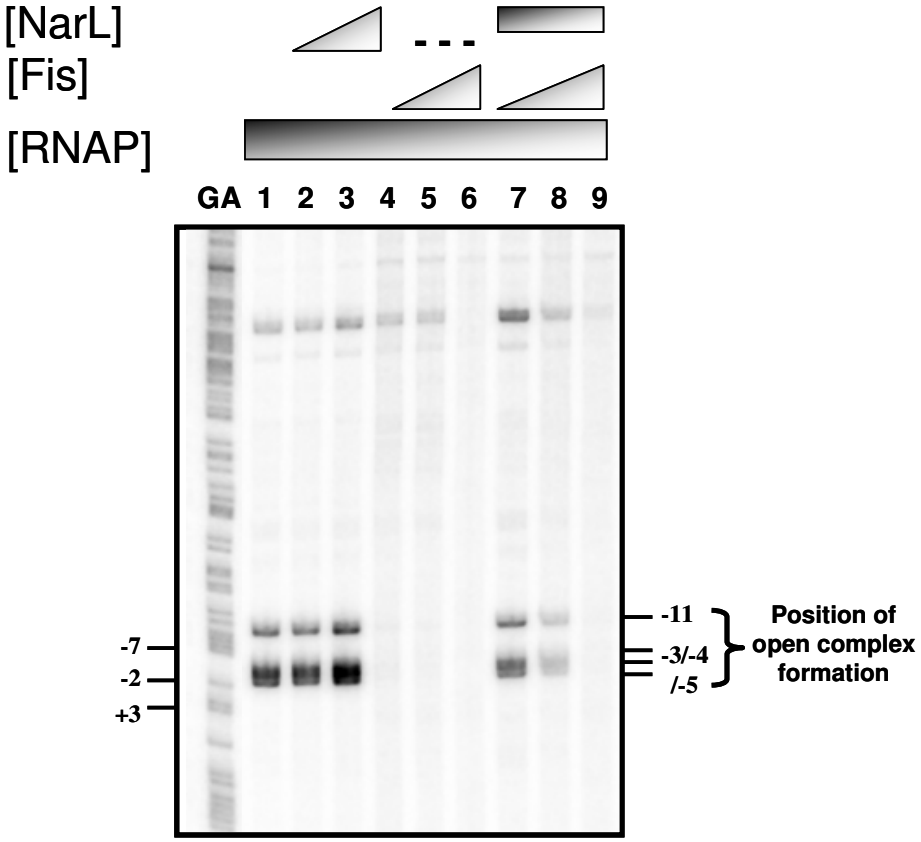

Fig 4.10b

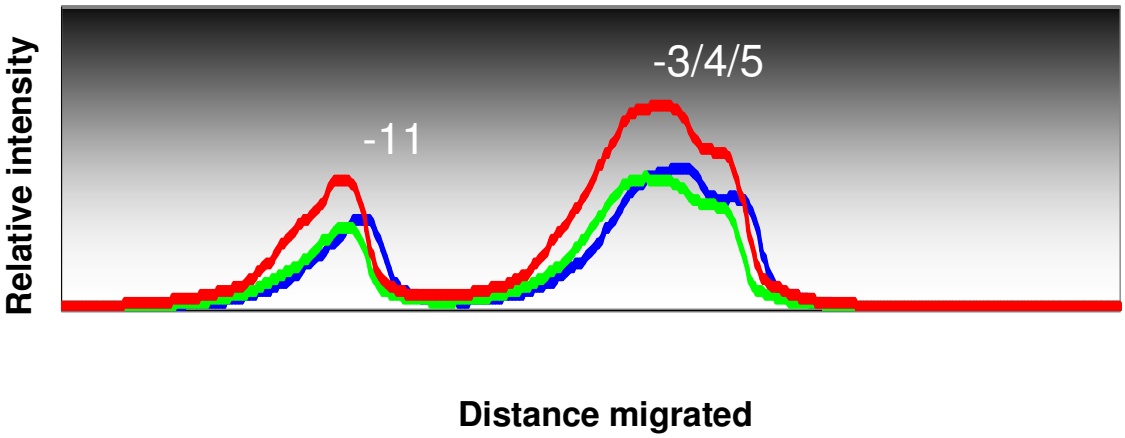

Figure 4.10 NarL enhances promoter opening at the *yeaR* promoter, while Fis represses

A. An *in vitro* potassium permanganate footprint experiments with purified NarL and Fis proteins was designed to show promoter opening in the presence and absence of DNA binding proteins. The end-labelled *yeaR100 Aat*II-*Hin*dIII promoter fragment was incubated with 50 nM of RNA polymerase (lanes 1 to 9) and varying concentrations of NarL and Fis purified protein and then subjected to potassium permanganate footprinting. The concentration of NarL in lanes 1-3 was 0, 0.2, and 0.4 μM, respectively, while NarL was present in lanes 7, 8 and 9 at 0.4 μM. The concentration of Fis was as follows: lane 4 and 7, 0.11 μM; lane 5 and 8, 0.22 μM and lane 6 and 9, 0.45 μM respectively. End-labelled *yeaR100 Aat*II-*Hin*dIII fragment was incubated with holo-RNA polymerase (RNAP) and Fis protein and subjected to potassium permanganate footprinting. Gels were calibrated using Maxam-Gilbert 'G+A' sequencing reactions and the location of cleavage sites produced by potassium permanganate footprinting are shown. In the absence of RNAP, no promoter opening was observed (data not shown).

B. Increased promoter opening in the presence of NarL was quantified by the measuring the increase in relative intensities of the bands located between positions -11 and -5, using Quantity-one software (BIORAD). Lanes one, two and three are represented by the green, blue red traces respectively.

Conversely, when the *yeaR100* promoter fragment was incubated with RNAP in the absence of NarL, but in the presence of increasing concentrations of Fis (lanes 4-6), a decrease in promoter opening is observed, suggesting that Fis inhibits the formation of the open complex by RNAP. This is probably due to the exclusion of RNAP, as Fis binding site II overlaps the -35 promoter element (fig. 4.2). The suggestion that Fis and NarL regulate the *yeaR* promoter by competing for binding was consistent with the decrease in open-complex formation when the promoter fragment was incubated with RNAP, a fixed concentration of NarL (0.4 μM) and increasing concentrations of Fis (lane 7-9). The formation of the open-complex is clearly inhibited as Fis is titrated into the reaction, confirming that Fis represses *yeaR* in competition with NarL activation.

The evidence provided by both *in vivo* and *in vitro* experiments, investigating the effect of NarL and Fis at the *yeaR* promoter, suggests that transcription initiation is controlled by a dynamic competition between the essential activator, NarL, which binds to a position proximal to the -35 element of the promoter, and a repressor, Fis, which binds to two overlapping sites. Steric hindrance excludes both the activator, NarL, and RNA polymerase from binding to the promoter.

Decreased nitrate activation of the *yeaR* promoter in aerobic conditons

As the activition of the *yeaR* promoter had been shown to be NarL-dependent, but independent of FNR, it was postulated NarL-dependent nitrate activation would lead to high expression from the *yeaR* promoter in aerobic cultures. To test this hypothesis, the *yeaR* promoter-*lacZ* fusion was transformed into the parental strain, JCB387, *fnr* and *fis* null derivatives and an *fnr fis* double mutant. Transformants were grown at 37°C in minimal medium, in the presence and absence of nitrate and nitrite. At all times cultures were aerated by vigorous shaking and were lysed and assayed at an OD_{650} of less than 0.3 to ensure that cultures were still growing aerobically (fig 4.11). Surprisingly, despite observing some nitrate

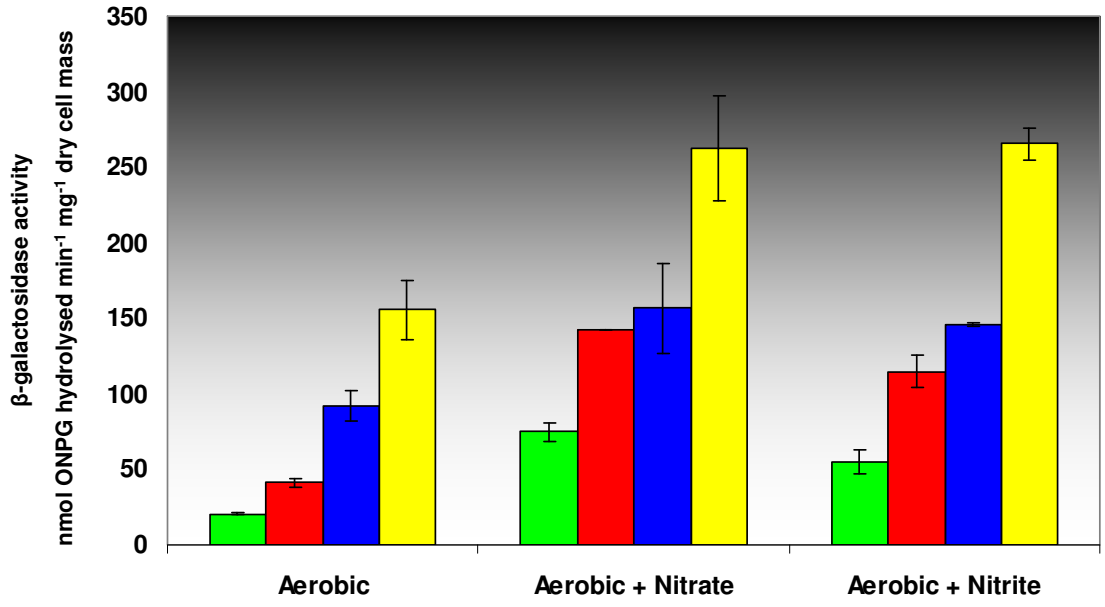

Figure 4.11 The effect of FNR and Fis on aerobic nitrate and nitrite transcription activation at the *yeaR* promoter

Expression from the *yeaR100* promoter, cloned as a promoter::*lacZ* fusion in pRW50, was measured by β-galactosidase activity assay in strains JCB387 (parental strain) (green), JCB387Δ*fis* (red), JCB387Δ*fnr* (blue) and JCB387Δ*fnr*Δ*fis* (yellow). Transformants were grown in minimal medium in the presence or absence of nitrate (20 mM) or nitrite (2.5 mM). At an OD_{650} of 0.6, cells were harvested, lysed and assayed for β-galactosidase activity.

and nitrite induction, the overall activity of the promoter was approximately 10-fold lower in aerobic conditions compared to anaerobic (see table 4.1). Interestingly, the deletion of *fnr* had a similar effect on the aerobic expression pattern of *yeaR* as in anaerobic conditions leading to an increase in the nitrite activation, but the overall activity of the promoter was still very low. The deletion of *fis* led to a similar increase in activity, but levels remained low, indicating that the suppression of promoter activity in exponentially growing aerobic cultures was not due to greater Fis repression. Finally, the double *fnr fis* mutant showed a similar increase in expression of the promoter in nitrate and nitrite as observed in anaerobic cultures, but again activity was approximately 10-fold lower in comparison to anaerobic promoter activity. In all strains and conditions tested, the activity of the *yeaR* promoter in aerobic conditions was between 10 and 20 fold lower than in anaerobic conditions.

The effect of NsrR on the activity of the *yeaR* promoter in aerobic conditions

A surprising difference in the overall activity of the *yeaR* promoter in aerobic and anaerobic conditions was observed. During aerobic growth, the global regulator of anaerobic growth, FNR, is inactive due to the oxidised redox state of its coordinated iron-sulphur centre. This means that no FNR-dependent promoters would be expressed in the presence of oxygen. These FNR-dependent promoters include those that control the expression of the nitrate and nitrite reductases, which would therefore not be expressed. This would mean hat no nitrite would be reduced and, as a consequence, no endogenous NO would be produced. As the *yeaR* promoter has been shown to be repressed by NsrR, the nitric oxide sensitive repressor, in transcriptomic investigations, it was reasoned that in aerobic cultures, when nitrite is not reduced, no endogenous NO is produced and therefore NsrR is not de-repressed. This might account for the lack of aerobic *yeaR* promoter activity.

To test this hypothesis, the β-galactosidase activity expressed from the *yeaR-lacZ* promoter fusion was measured in the parental strain, JCB387, and the *nsrR* null derivative,

JCB3901 (figure 4.12). Cultures were grown in minimal medium in the presence and absence of nitrate. Cultures were aerated by vigorous shaking at 37°C and harvested at an OD_{650} of less than 0.3. The activity of the promoter in the parental strain, in aerobic conditions, was again shown to be 40 fold lower than in anaerobic conditions (table 4.1). The deletion of *nsrR* led to a 6-fold increase in promoter activity in both the presence and absence of nitrate in comparison to the parental strain. However, as with other strains, the activity of the promoter in aerobic conditions was 10-fold lower than in anaerobic conditions in the presence of nitrate, showing that NsrR is not responsible for the apparent aerobic repression of p*yeaR*. This result was anaomalous to the findings of Lin *et al*., (2007), who reported nitrate-dependent aerobic promoter activity, in an *nsrR* mutant, at comparable levels to anaerobic conditions in the presence of nitrate. The results of Lin *et al.,* (2007) would suggest that NsrR controls anaerobic expression of *yeaR* in response to anaerobically produced NO. However, this conclusion is not reflected by the results of this study.

Aerobic nitrate activation of p*yeaR* is NarL-dependent

Although it was demonstrated that the overall activity of the *yeaR* promoter was approximately 10-fold during aerobic growth, a 6-fold activation was observed when nitrate was included in the aerobic growth medium. This suggested that NarL still activated the *yeaR* promoter in aerobic cultures. To test that this effect was NarL dependent, the *yeaR-lacZ* promoter fusion was used to transform the parental stain, JCB387, and derivatives mutated in *narL, narP* and a *narLP* double mutant. Transformants were grown in aerated minimal medium in the presence and absence of nitrate, lysed and assayed at an OD_{650} of less than 0.3 (figure 4.13). A 4 to 5 fold increase in promoter activity was observed in the parental strain when nitrate was added to the medium. A similar effect was seen in the *narP* null derivative, suggesting that NarP is not required for activation of this promoter. Conversely, in the *narL* and *narLP* null derivatives, activity of the promoter was not increased in the presence of

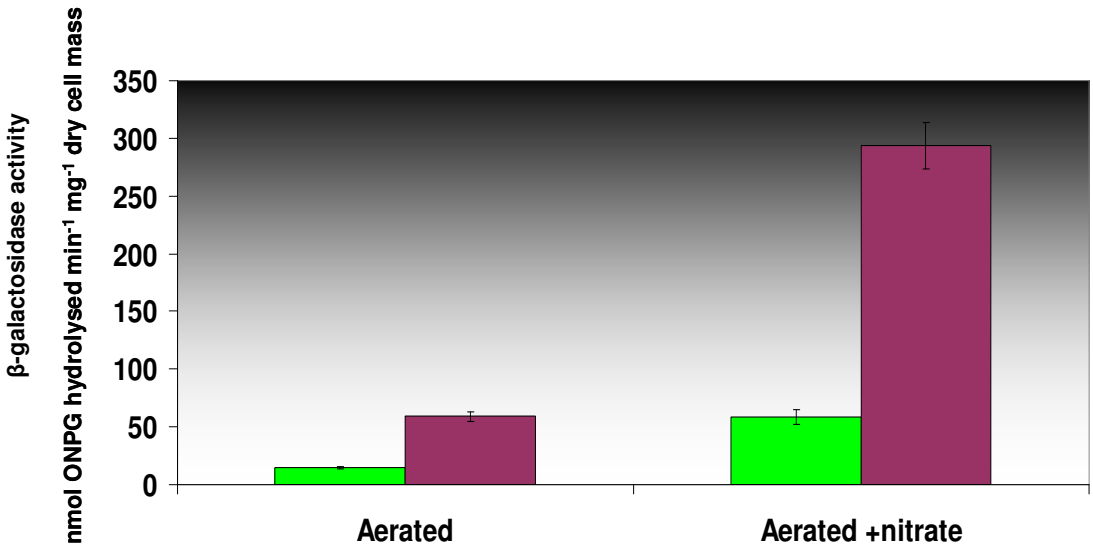

Figure 4.12 NsrR repression of aerobic nitrate activation of the *yeaR* promoter

The activity of the *yeaR-lacZ* promoter fusion was measured by β-galactosidase activity assay in strains JCB387 (green), and JCB3901 (JCB387Δ*nsrR*) (claret) grown aerobically in the absence and presence of 20 mM nitrate. The cultures were lysed and assayed at an OD_{650} of < 0.3 to ensure oxygen was still abundant in the culture.

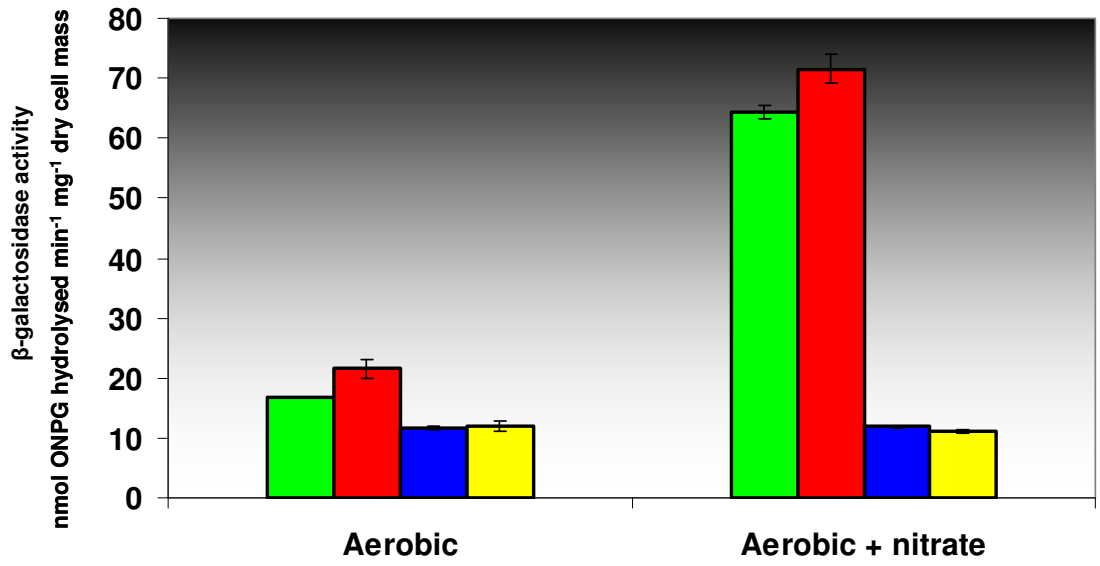

Figure 4.13 NarL-dependent aerobic nitrate activation of the *yeaR* promoter

The activity of the *yeaR-lacZ* promoter fusion was measured by β-galactosidase activity assay in strains JCB387 (green), JCB3875 (Δ*narP*) (red), JCB3883 (Δ*narP*) (blue) and JCB3884 (Δ*narLP*) (yellow). Transformants were grown aerobically in the absence and presence of 20 mM nitrate. At an OD650 < 0.3, cells were harvested, lysed and assayed for β-galactosidase activity.

nitrate, confirming that NarL is still required for aerobic nitrate activation of the *yeaR* promoter.

Nitrate activation of the *E. coli ogt* promoter is NarL dependent

In order to confirm the findings of Meng Xu (PhD thisis, Birmingham), that expression from the *ogt* promoter in response to nitrate is NarL dependent, the *ogt100* fragment was cloned as a promoter-*lacZ* fusion into the plasmid, pRW50. This construct was then transformed into the parental strain, JCB387, and a Δ*narL* derivative, JCB3883. Transformants were grown anaerobically at 37°C in minimal medium in the presence and absence of nitrate until they reached mid-exponential growth phase. At this point the cells were harvested, lysed and assayed for β-galactosidase activity (fig 4.14). In the absence of nitrate, a low basal level of activity was observed in both the parental and mutant strain. In the presence of nitrate there was a dramatic 4.5-fold increase in promoter activity in the parental strain. However, this increase was not observed in the *narL* mutant confirming that activation of this promoter is NarL-dependent.

Transcription activation of the *ogt* promoter in response to nitrate is dependent on NarL binding to two sites in the promoter region

Previous studies by Meng Xu had identified a single binding site for NarL in the *ogt* promoter region and had shown, using EMSA and DNase I assays, that NarL binds directly to the promoter region at a site centred at position -78.5 with respect to the transcription start site (NarL I). Further investigation into the promoter structure using DNase I footprinting by Douglas Browning (University of Birmingham), identified a second binding site for NarL centred around -45.5 with respect to the transcription start site (NarL II). In order to determine the dependence of *ogt* promoter activity on these two NarL binding sites, a number of mutated derivative promoters were constructed in which specific residues had been altered in either the NarL I (*ogt102*) or the NarL II (*ogt104*) binding site, to decrease the similarity of the site to the consensus sequence, and therefore reduce NarL binding to the promoter region. These

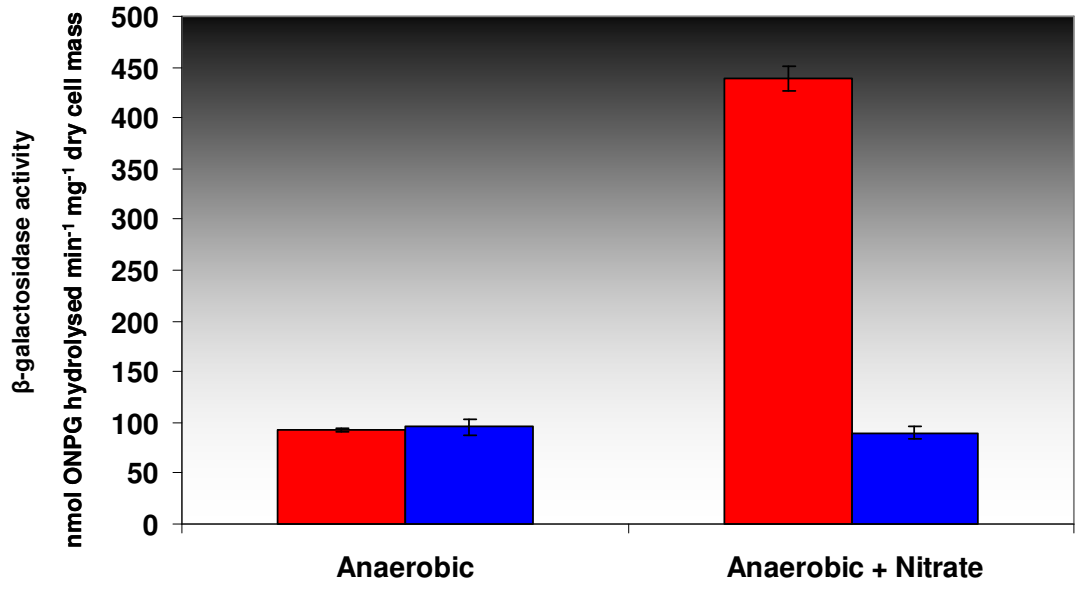

Fig 4.14 Nitrate induced expression of the *ogt* promoter is NarL dependent

The *ogt100* fragment was cloned as a promoter-*lacZ* fusion in the plasmid pRW50and used to transform strains JCB387 (parental strain) (red) and JCB3883 (JCB387Δ*narL*) (blue). Transformants were grown in minimal medium (MS) anaerobically in the presence or absence of nitrate (20 mM). At an OD650 ≈0.6, cells were harvested, lysed and assayed for β-galactosidase activity.

constructs were then cloned as *lacZ*-promoter fusions into pRW50. The activity of the promoter was measured after anaerobic growth in the presence and absence of nitrate and compared to that of the wild-type promoter-*lacZ* fusion, *ogt100* (fig 4.15). The nitrate dependent activity of the *ogt* promoter clearly requires binding of NarL to both sites as mutations that disrupt either site results in the loss of nitrate induction. The binding positions for NarL at -78.5 and -45.5 with respect to the transcription start site, and the dependence upon both sites for promoter activity, suggest that the *ogt* promoter is a class III NarL-dependent promoter and demonstrates the apparent versatility of NarL as a transcription activator.

The nucleoid associated protein, Fis, represses transcription from the *ogt* promoter *in vivo*

As with the *yeaR* promoter, a difference in activity from the *ogt* promoter was observed when the promoter activity was assayed in rich versus minimal medium, implicating Fis as a regulator of this promoter (fig 4.16). To assess the role of Fis at the *ogt* promoter, the p*ogt-lacZ* fusion was compared in the parental strain, JCB387, and the *Δfis* derivative, JCB3871 (fig 4.16). Results show that nitrate-induced activity from the ogt promoter is severely restricted during growth in rich medium (Lennox broth) supplemented with glucose and that this suppression is relieved in the *fis* null derivative. A similar trend of activity was observed for cultures grown in minimal medium supplemented with glycerol, indicating that Fis binds to and represses this promoter *in vivo*. Further *in vitro* investigations by Meng Xu and Douglas Browning have shown that Fis does indeed bind specifically to the *ogt* promoter at a single site centred around -82 with respect to the transcription start site (Squire *et al*., 2009). The position of the Fis binding site at the *ogt* promoter overlaps the upstream binding site for NarL, NarL I, suggesting that p*ogt* and p*yeaR* are regulated by a shared transcriptional mechanism, whereby NarL activation of the promoters, in response to nitrate, is repressed by Fis via competition for overlapping binding sites. Furthermore, the comparison of these two promoters show that NarL

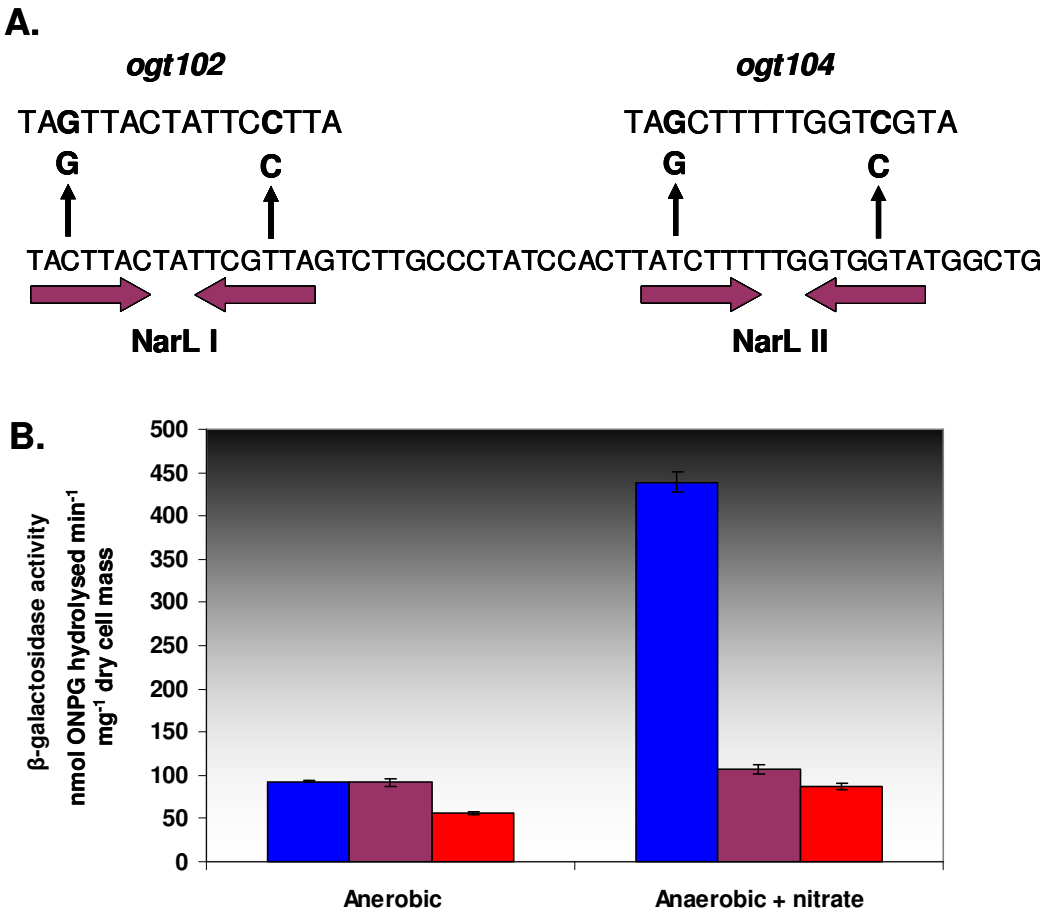

Fig. 4.15 Transcription from the *ogt* promoter is dependent upon NarL binding to two sites in the promoter region

A. Two mutant derivatives of the *ogt100* promoter fragment were created by Doug Browning (University of Birmingham) using mega primer PCR. Specific, conserved residues in the two NarL binding sites were changed to decrease the similarity of the NarL binding site to the consensus sequence. The promoter fragment that was mutated in the upstream NarL site, NarL I, was designated *ogt102* while the promoter fragment that was mutated in the downstream NarL binding site, NarL II, was designated *ogt104*.

B. Mutated *ogt* promoter derivatives, *ogt102* (burgundy) and *ogt104* (red), were cloned as *lacZ*-promoter fusions in pRW50. These were then used alongside the wild-type promoter fusion, *ogt100* (blue), to transform parental strain JCB387. Transformants were grown at 37℃ without shaking to an $O.D_{650}$ of approximately 0.6, in minimal medium in the presence and absence of 20 mM nitrate, at which point cells were lysed and assayed for β-galactosidase activity.

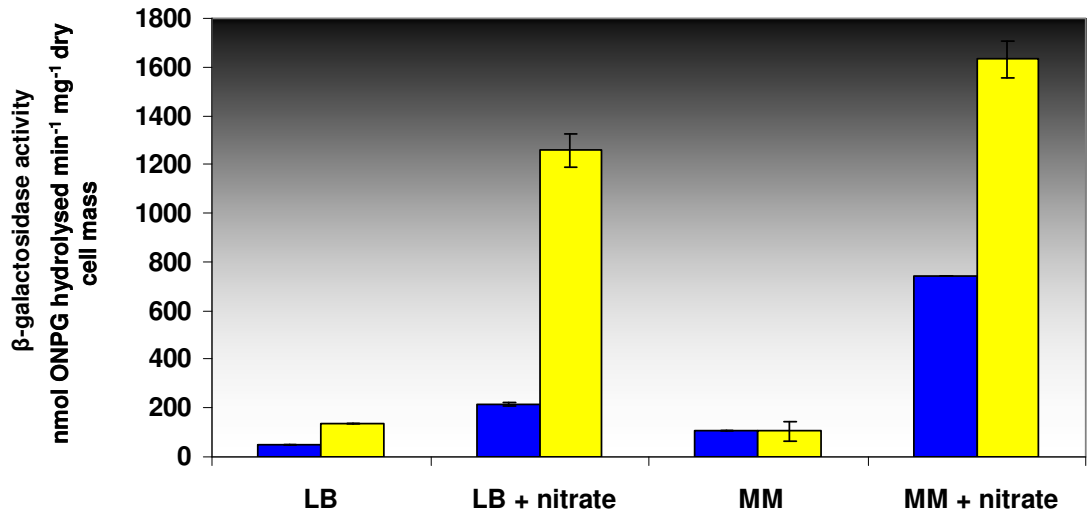

Fig. 4.16 The effect of Fis on transcription activation at the *ogt* promoter

Expression from the *ogt100* promoter fragment, cloned as a promoter*::lacZ* fusion in pRW50, was measured by β-galactosidase assay in strains JCB387 (parental strain) (blue) and JCB387Δ*fis* (yellow) . Transformants were grown in in rich medium (Lennox broth) and minimal medium (MS) anaerobically in the presence or absence of nitrate (20 mM). At an OD_{650} of 0.6, cells were harvested, lysed and assayed for β-galactosidase activity.

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is apparently able to function as both a class II transcription activator at p*yeaR* and a class III transcription activator at p*ogt*.

Chapter 5

Results

Indirect FNR regulation of NarL dependent promoters, yeaR and ogt.

Introduction

It has been previously established that the regulation of anaerobic nitrate and nitrite reduction in *E. coli* usually requires the activity of the global regulator of anaerobiosis, FNR, and the nitrate and nitrite responsive dual acting two component systems NarXL and NarQP. At all previously studied promoters, FNR and the response regulator components, NarL and NarP, work synergistically to activate transcription at target promoters. The anaerobic activity of FNR is governed by the redox state of an associated iron-sulphur cluster, which is sensitive to oxygen and the reactive nitrogen species, nitric oxide (Crack *et al.*, 2008a; Cruz-Ramos *et al.*, 2002). Further control of the synthesis of the nitrate and nitrite reductase genes is provided by the response regulators, NarL and NarP, whose activity is dependent upon the action of the associated sensor kinases, NarX and NarQ, which auto-phosphorylate in response to the presence of nitrate and nitrite (Stewart, 1993). During its life cycle, *E. coli* has to be able to detect and respond to not only the presence and absence of oxygen, nitrate and nitrite, but also be sensitive to the relative abundance of these alternatives. The ability to 'fine tune' expression of nitrate and nitrite-dependent promoters is attributed in many cases to the action of the sensor kinase components, NarX and NarQ, which demonstrate different sensitivities to both nitrate and nitrite, and elicit different effects in response to both the species of nitrogen-oxide and the concentration at which it is present (Lee *et al.*, 1999; Stewart, 1993). However, the most significant property of the sensor-kinase, NarX, is that in the presence of nitrite, it has been shown to reverse its function and actively dephosphorylate phospho-NarL, counteracting the NarL-kinase activity of NarQ, resulting in lower levels of NarL dependent gene expression (Stewart, 1993).

Two promoters driving the expression of the gene, *ogt,* and the operon, *yeaR-yoaG*, have been shown in the previous chapter to be activated in response to nitrate and nitrite by a NarL dependent mechanism. In both cases, the activation by NarL is countered by the nucleoid associated protein, Fis, which antagonizes NarL activity competing with NarL for overlapping binding sites at both promoters (Squire *et al.*, 2009). In all conditions tested, nitrate and nitrite induced activity of both the *yeaR* and *ogt* promoters required functional NarL, however under certain conditions, the absolute activity of the promoter changed in response to the presence or absence of functional FNR, which had been predicted by transcriptomic studies to be a repressor of the *yeaR* promoter (Constantinidou *et al.*, 2006). Initial investigations into the activity of the *yeaR* promoter, in the absence of functional FNR, showed that NarL-dependent activation of the promoter was FNR independent, but nitrite induction of the promoter was increased 5-fold in the FNR null strain (fig 4.7). Despite the apparent regulation of *yeaR* by FNR in both this study and transcriptomic studies, no putative binding site for FNR, with significant similarity to the consensus sequence, has been identified in the *yeaR* promoter region.

In this chapter, the role of FNR in increased NarL-dependent nitrite induction of the *yeaR* promoter, and whether this effect was caused by direct binding of FNR to the promoter region or another indirect mechanism, was investigated.

Purified FNR binds to the *yeaR* promoter region with low affinity

It has been previously demonstrated that the activity of the *yeaR* promoter is 3 to 4-fold higher in the presence of nitrite, when assayed in an FNR null derivative, when compared to the parental strain (fig 4.6). In order to determine whether this is a direct effect of FNR binding to the *yeaR* promoter region, the *yeaR100 Eco*RI-*Hin*dIII promoter fragment was radio-labelled for use in an electro-mobility shift assay. Purified promoter DNA was incubated with increasing concentrations of purified FNR D154A and DNA-protein complexes were separated

by PAGE (Fig 5.1). At FNR concentrations of 1 μM and above, some DNA-protein complexes formed, causing a band shift. However, the affinity of FNR for this promoter was very low. As there was some indication that FNR could bind to p*yeaR* with low affinity, the promoter sequence was re-investigated in

an attempt to identify any putative FNR binding sites with some resemblance to the consensus sequence. Subsequently, two putative FNR binding sites, with 6 bases out of 10 that matched the consensus sequence and were located in likely repressor positions, were identified in the promoter region. These FNR sites were identified proximal to the NarL binding site and were centred at positions -22.5 and -63.5 with respect to the transcription start site, and were designated sites FNR 1 and FNR 2 respectively.

Site directed mutagenesis of putative FNR sites 1 and 2

Site directed mutagenesis was used to remove any resemblance of the putative FNR sites to the FNR consensus sequence. At FNR site 1, the downstream half site ATCAG, which matched the consensus sequence, ATCAA, at 4 out of 5 bases was mutagenised to CATTG to remove all similarity to the consensus sequence (Fig 5.2). In the case of FNR 2, specific nucleotide changes were made to destroy simultaneously the putative FNR site and create a truncated promoter fragment by introducing a *Bam*HI site at FNR 2. These mutagenised promoters, designated YEAR102 and YEAR200 respectively, were digested with *Eco*RI-*Hin*dIII (YEAR102) or *Bam*HI-*Hin*dIII (YEAR200), and ligated into the *lacZ* fusion vector, pRW50, to make plasmids pYEAR102 and pYEAR200, respectively.

FNR represses the *yeaR-yoaG* indirectly *in vivo*

The mutagenised promoter-*lacZ* fusions, pYEAR102 and pYEAR200 were transformed into the wild type strain, JCB387, and its FNR null derivative, JCB3911, and grown anaerobically in the presence and absence of nitrate or nitrite. The activity of each promoter in each strain was determined by β-galactosidase activity assay (Fig 5.3ai and bi). Destroying putative FNR site 1

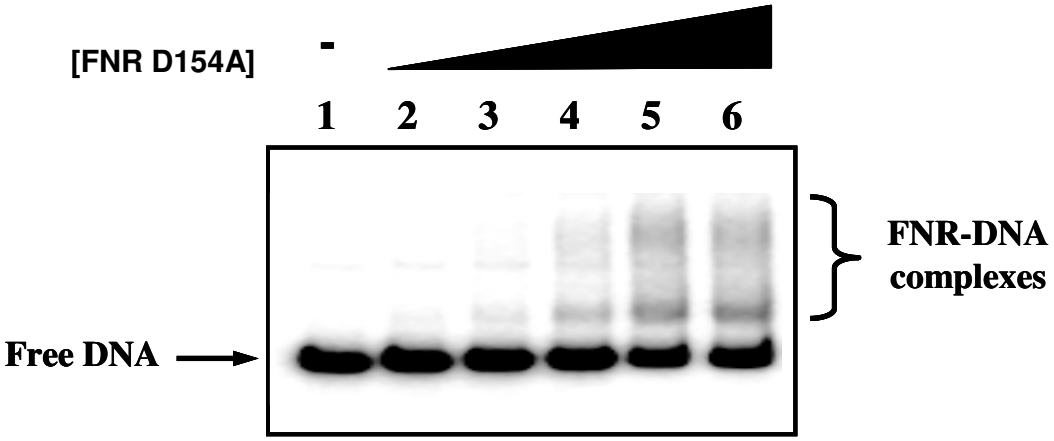

Figure 5.1 FNR binds to the *yeaR-yoaG* promoter with low affinity

End-labelled *yeaR-yoaG* fragment was incubated with increasing concentrations of FNR D154A and protein-DNA complexes were separated by polyacrylamide gel electrophoresis. The concentration of FNR D154A in lanes 1-6 was 0 μM, 0.25 μM, 0.5 μM, 1 μM, 2 μM and 3 μM respectively.

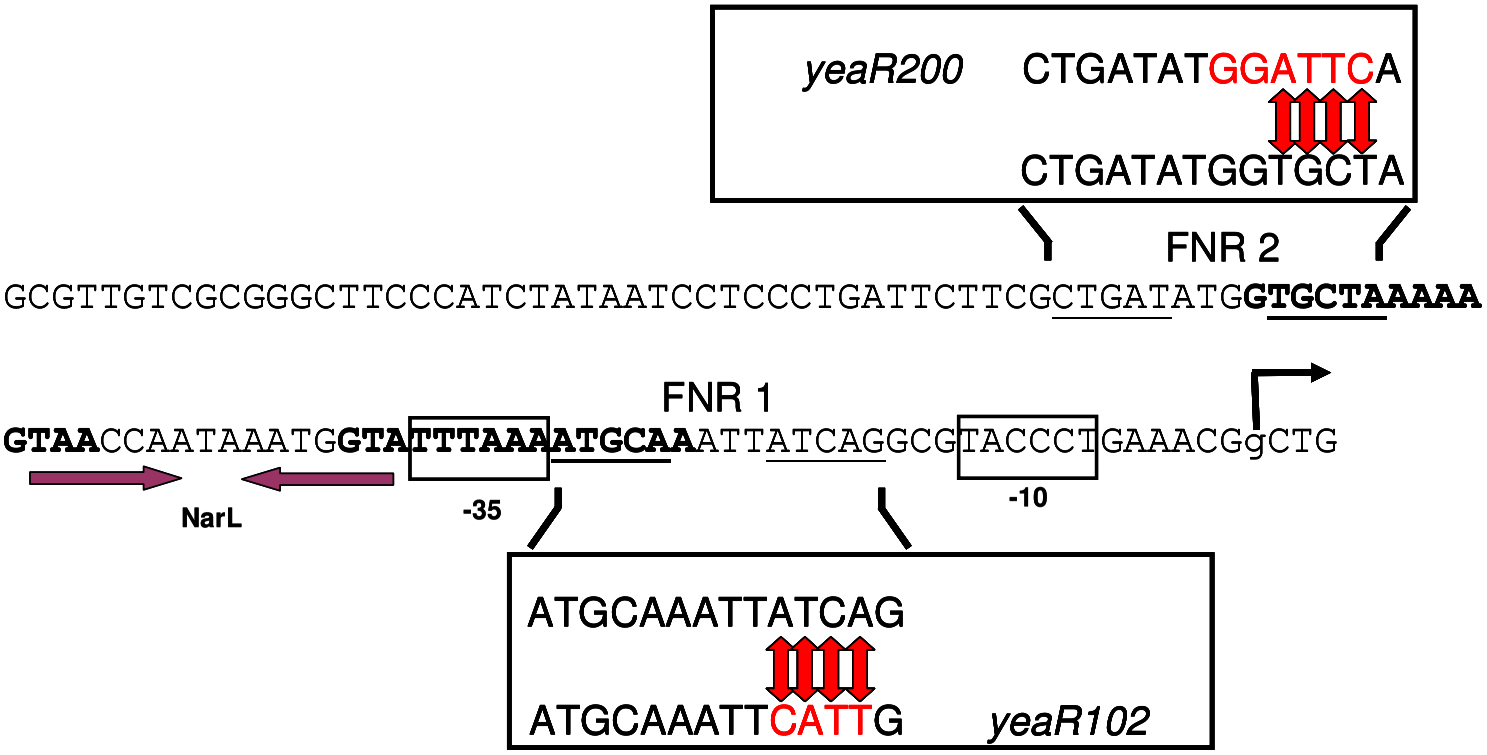

Figure 5.2 Mutagenesis of putative FNR binding sites, FNR1 and FNR 2 in the *yeaR-yoaG* promoter region

Two putative FNR binding sites with 60% identity to the FNR consensus sequence were identified in the *yeaR-yoaG* promoter region. The putative FNR site centered around -22.5nt with respect to the transcription start site was designated FNR 1. The putative FNR site centered around -62.5 with respect to the transcription start site was designated FNR 2. Using site-directed mutagenesis, specific mutations were introduced to each site to destroy any similarity to the FNR consensus sequence. At FNR 2, specific mutations were introduced to both half-sites to remove any similarity to the FNR consensus sequence and concurrently introduce a *Bam*HI site, producing a truncated promoter fragment. The -10 and -35 promoter elements, NarL binding site and Fis binding sites are indicated by the black boxes, purple arrows and bold text, respectively.

Fig 5.3Ai

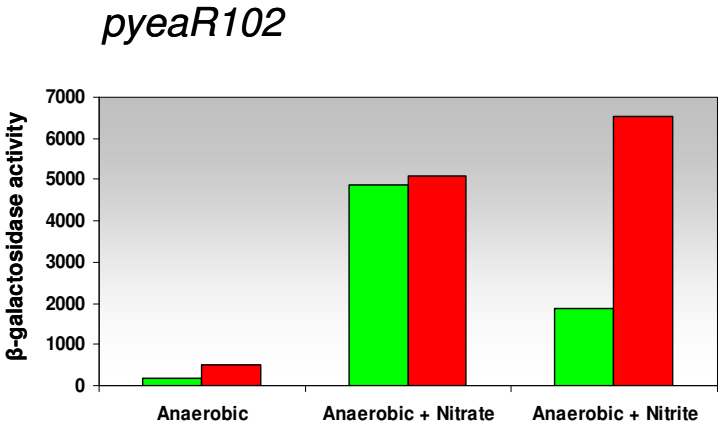

Fig 5.3Aii

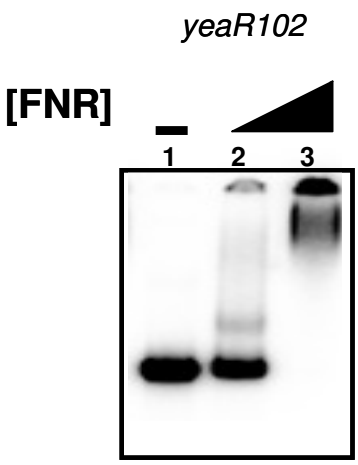

Fig 5.3Bi

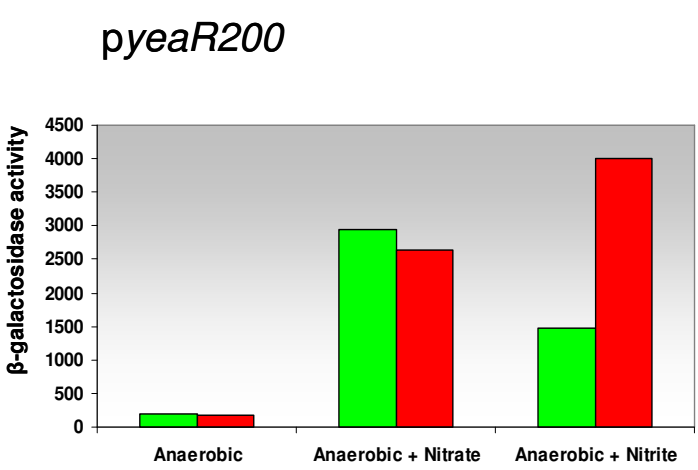

Fig 5.3Bii

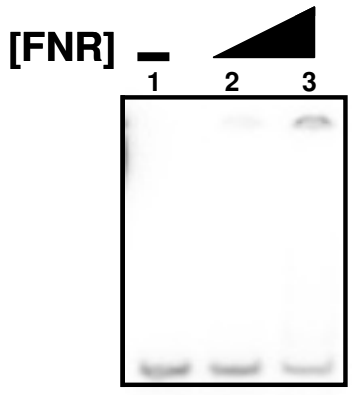

Fig. 5.3 A and B. FNR repression of the *yeaR-yoaG* promoter is indirect

Fig 5.3Ai and 5.3Bi. Strains JCB387 (green) and JCB3911 (Δ*fnr*) (red) were transformed with mutagenised plasmids, *pyeaR102* (Ai) and *pyeaR200* (Bi) and grown in defined minimal medium anaerobically in the presence and absence of nitrate (20 mM) or nitrite (2.5 mM). At an OD650 ≈0.6, cells were harvested, lysed and assayed for β-galactosidase activity. Units are expressed as nmol ONPG hydrolysed min^{-1} mg^{-1} dry cell mass.

Fig 5.3Aii and 5.3Bii. Mutagenised promoter fragments *yeaR102* and *yeaR200* were digested with *Eco*RI-*Hin*dIII and *Bam*HI-*Hin*dIII respectively, and end-labeled. Fragments were incubated with increasing concentrations of FNR D154A and protein-DNA complexes were separated by polyacrylamide gel electrophoresis. Lanes 1, 2 and 3 contain zero, 1 and 3 μM FNR D154A respectively.

(pYEAR102) caused an overall increase in activity in all anaerobic conditions when compared to the wild type promoter in a wild-type strain (data not shown). However the activity of pYEAR102 still exhibited the same pattern of increased activity as the wild type promoter (significant increase in the presence of nitrite) when assayed in an FNR mutant (Fig 5.3ai). This suggested that FNR 1 is not the binding site for FNR at the *yeaR-yoaG* promoter. The increase in overall promoter activity observed when FNR site 1 was mutagenised may be due to interference with the overlapping repression site for Fis. When assayed in a wild type bacterium, the activity of pYEAR200, the *yeaR-yoaG* promoter mutated and truncated at FNR 2, was comparable to the wild-type promoter, pYEAR100 (data not shown). This suggested that the truncated promoter includes all necessary promoter elements for transcription activation. However, the truncated promoter still showed an increase in activity in all conditions, most dramatically during growth in the presence of nitrite, when assayed in an FNR mutant strain, suggesting that FNR does not bind at putative FNR site 2, and any FNR effect on the truncated promoter was indirect as the truncated promoter behaves in the same way as the natural promoter in the parental strain (fig. 5.3bi).

Purified FNR does not bind to FNR 1 or FNR 2 *in vitro*

The mutagenised *yeaR-yoaG* promoter fragments, YEAR102 and YEAR200 were end-labelled and incubated with FNR D154A. The YEAR102 fragment, mutagenised at FNR 1, still bound FNR at concentrations comparable to the wild type promoter. This evidence suggests that FNR does not bind at putative FNR site, FNR 1 (Fig. 5.3Aii). Finally the truncated, end-labelled YEAR200 fragment (*Bam*HI-*Hin*dIII) was incubated with purified FNR D154A and protein DNA complexes were separated by PAGE. No band shift was observed at any concentration tested, confirming the conclusion that FNR does not bind within the truncated promoter (fig. 5.3bii). By interpreting the *in vivo* and *in vitro* data together it can be concluded that any FNR repression effect on the activity of the *yeaR-yoaG* promoter is likely to be indirect.

A common feature of increased nitrite induction of NarL-dependent promoters in an FNR mutant strain

In the previous chapter, it was established that the *yeaR* and *ogt* promoters were co-regulated by a common mechanism in response to nitrate. The activity of both *yeaR* and *ogt* was entirely dependent upon functional NarL and the activation by NarL was countered by the binding of Fis by competitive binding for overlapping sites and the exclusion of an essential activator. Comprehensive studies of the *yeaR* promoter have shown that the activation in response to nitrite is 3-fold higher in a strain lacking functional FNR, suggesting an FNR repression effect at the promoter; however this effect has been shown to be indirect. As the *ogt* promoter is regulated in a very similar fashion to the *yeaR* promoter, the effect of an FNR mutation on the nitrite induction of both promoters was compared.

The *yeaR100* and *ogt100* promoter-*lacZ* fusions were used to transform a parental strain, JCB387, and an *fnr* null derivative strain, JCB3911. Transformants were grown anaerobically in minimal medium in the presence and absence of nitrate (20 mM) and nitrite (2.5 mM) until cultures reached mid-exponential growth phase. At this point the cells were harvested, lysed and assayed for promoter-driven β-galactosidase activity (fig 5.4). In the case of the *yeaR* promoter (A), the activity of the promoter in response to nitrite was 5-fold higher in the absence of functional FNR than in the wild-type strain. The same effect was seen at the *ogt* promoter, albeit at lower absolute levels (B), where a 4-fold increase in promoter activity in response to nitrite was observed in the FNR null derivative. The fact that both promoters, which have shared regulatory features, are affected in the same way in response to nitrite in an FNR null strain suggests a common indirect mechanism that increases NarL-dependent promoter activity in these conditions.

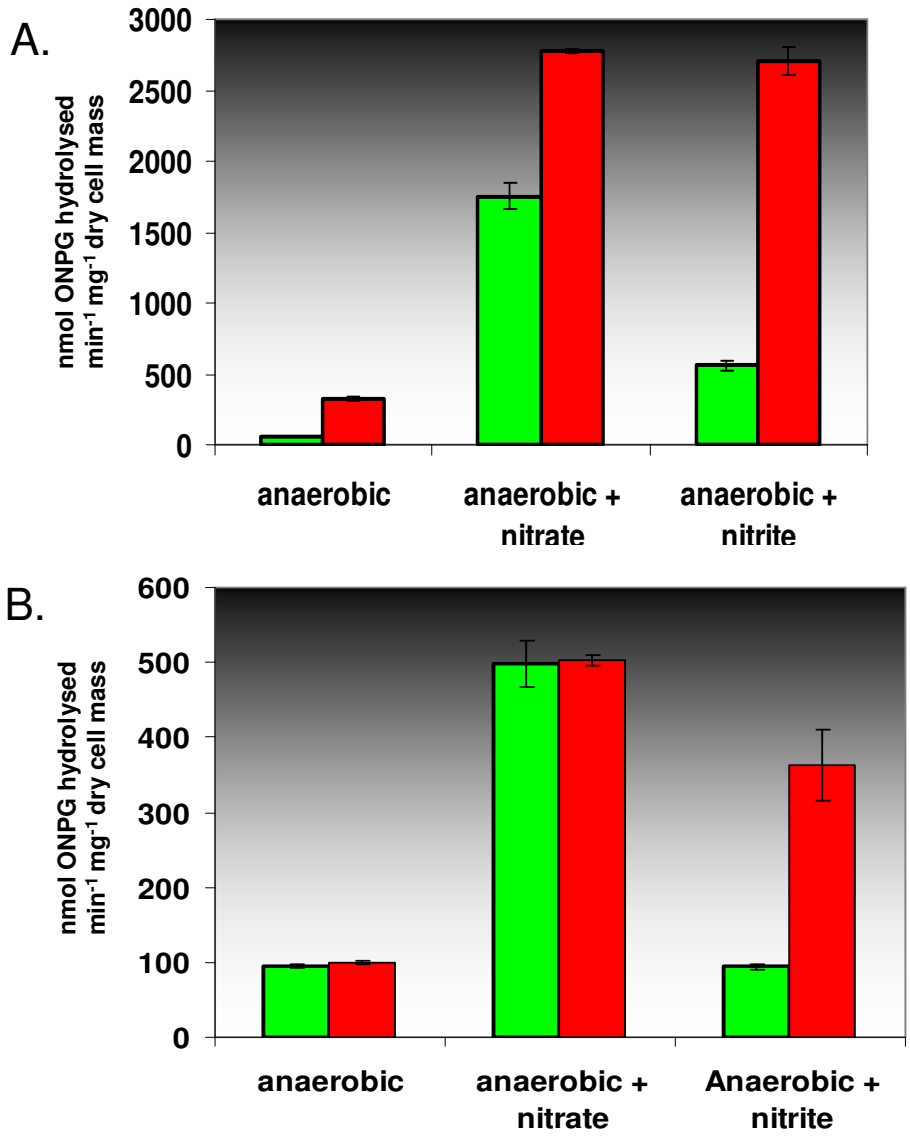

Figure 5.4 The effect of an *fnr* mutation on nitrate and nitrite activation of the *yeaR* and *ogt* promoters.

Strains JCB387 (parental strain) (green) and JCB3911 (JCB387Δ*fnr*) (red), were transformed with plasmid YEAR100 (Chart **A**) and OGT100 (Chart **B**) and grown in minimal medium (MS), anaerobically in the presence or absence of nitrate (20 mM) or nitrite (2.5 mM). At an OD650 ≈0.5, cells were harvested, lysed and assayed for β-galactosidase activity. For all β-galactosidase activity assays, units are expressed as nmol ONPG hydrolysed min^{-1} mg^{-1} dry cell mass. In this and all subsequent experiments, all assays were duplicated: data are the mean and standard error of the mean from three independent cultures.

Increased nitrite dependent activation of the *yeaR* promoter in an FNR mutant is NarL dependent.

In vivo and *in vitro* investigation of the *yeaR* promoter region has established that NarL was required for promoter activation. NarL has been shown to bind to the *yeaR* promoter to a 7-2-7 inverted repeat sequence located at -43.5 with respect to the transcription start site. Surprisingly, despite the 7-2-7 binding arrangement of the NarL site, a conformation that should allow for binding of the homologous response regulator, NarP, no binding of NarP to the promoter has been shown and little effect on promoter activity in a *narP* null strain was observed (table 4.1). This was especially surprising as, in the presence of nitrite, levels of phospo-NarL in comparison to phospho-NarP would be reduced by NarX-phosphatase activity. Although very little effect of an *nsrR* mutation on promoter activity *in vivo* was observed in this study (fig 4.7), transcriptomic studies do infer a repression effect of NsrR at the *yeaR* promoter and a putative binding site has been identified in the promoter region. It was postulated that under conditions where nitrite alone is present, nitric oxide would build up to a level that would de-activate NsrR and FNR and only under these specific conditions it could be possible, though unlikely, for NarP to activate the *yeaR* promoter.

In order to confirm that the increase in nitrite-induction of the *yeaR* promoter in a FNR null strain was not due to increased NarP activity, the *yeaR* promoter-*lacZ* fusion was used to transform a wild type strain and strains lacking in functional *fnr, narL, narLP, fnr nsrR narL,* and *fnr nsrR narLP*. Transformants were grown anaerobically in minimal medium in the presence or absence of nitrate or nitrite until mid-exponential growth phase was reached. At this point cells were harvested, lysed and assayed for β-galactosidase activity (fig 5.5). As in previous experiments, the effect of an *fnr* mutation was an increase in the observed nitrite induction of the *yeaR* promoter (pink bars) in comparison to the wild-type (red bars). In strains lacking in NarL, but where FNR and NsrR are still functional (green and navy) the promoter was un-inducible in any condition, confirming that NarL is required for activation of this

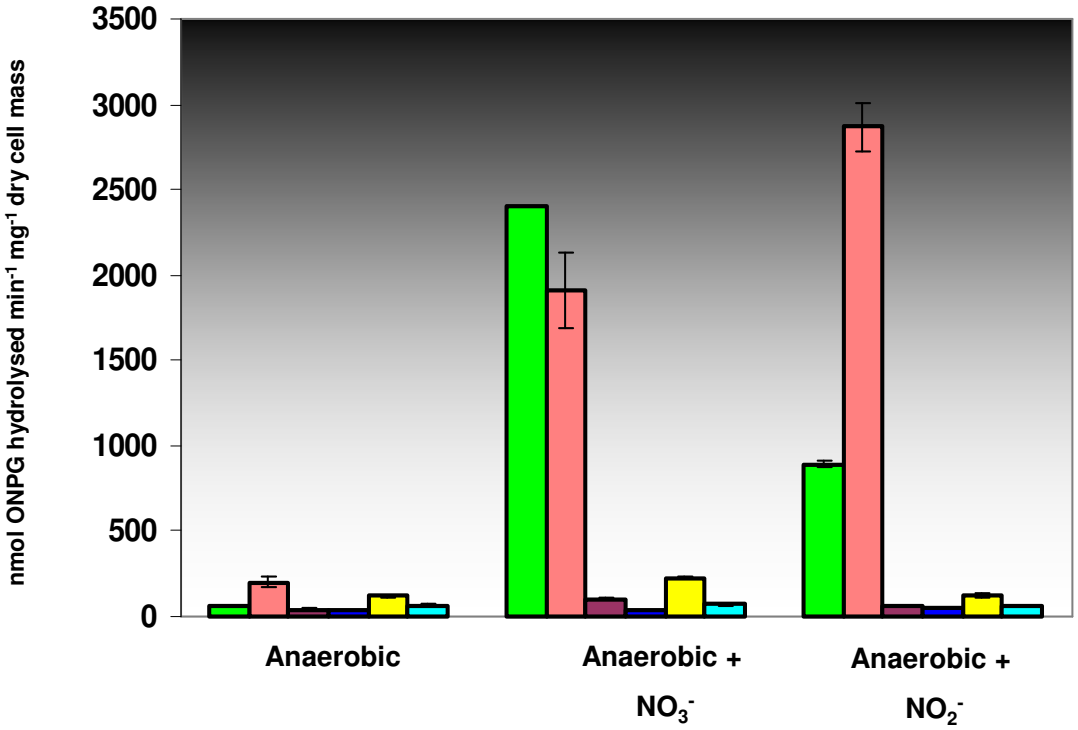

Figure 5.5 Nitrate and nitrite activation of the *yeaR* promoter in an *fnr* mutant requires NarL

The *yeaR100* promoter fragment was cloned as a *lacZ-promoter* fusion in pRW50 and transformed into the parental strain, JCB387 (green), and strains lacking functional *fnr nsrR* (pink), *narL* (claret), *narLP* (navy), *fnr nsrR narL* (yellow) and *fnr nsrR narLP* (turquoise). Transformants were grown anaerobically in the presence and absence of nitrate or nitrite, harvested, lysed and assayed for β-galactosidase activity

promoter in these conditions. When *fnr* and *nsrR* are mutated (yellow and turquoise), the activity of the promoter was not increased by the presence of nitrate or nitrite through the action of NarP (yellow) demonstrating that *yeaR* promoter activity and the apparent FNR effect are absolutely dependent upon NarL.

Increased NarL dependent nitrite induction of the *yeaR* promoter in an FNR null mutant is not due to a secondary Fis effect

The previous chapter established the mechanism for the regulation of the *yeaR* promoter by competition between NarL dependent activation and Fis dependent repression. It has been established that cellular Fis levels fluctuate considerably during growth and peak during early-exponential growth phase (Ali Azam *et al.*, 1999). As would be expected, the growth of the *fnr* null mutant strain was severely inhibited during anaerobic growth in the presence of nitrate and nitrite, even when alternative electron acceptor TMAO was present in the medium (the expression of the TMAO reductases is FNR independent), meaning that the relative Fis levels in the *fnr* null strain may differ from the wild-type at the point at which promoter activity is measured due to growth rate differences.

In order to ascertain whether the increased nitrite-dependent activation of the *yeaR* promoter in an *fnr* null strain is due to a secondary Fis effect, a double *fnr*$^{-}$ *fis* strain was constructed by transducing the JCB387Δ*fnr::*Cm^{R} mutant, JCB3911, to streptomycin resistance with P1 phage propagated on the JCB387Δ*fis::*Stp^{R} strain, JCB3871. The wild type (green), *fis* (blue), *fnr* (red) and *fis fnr* (yellow) strains were transformed with the *yeaR100* promoter-*lacZ* fusion and grown anaerobically in minimal medium in the presence and absence of nitrate and nitrite (fig 5.6). All cultures were grown to an optical density (650 nm) of 0.5 (± 0.05) at which point cells were harvested, lysed and assayed for β-galactosidase activity. In the wild type strain (green), the normal profile for promoter activity was observed, with highest promoter activity reported in the presence of nitrate and only limited activity in the presence of nitrite. As observed previously, the absolute activity of the promoter in the absence of functional Fis

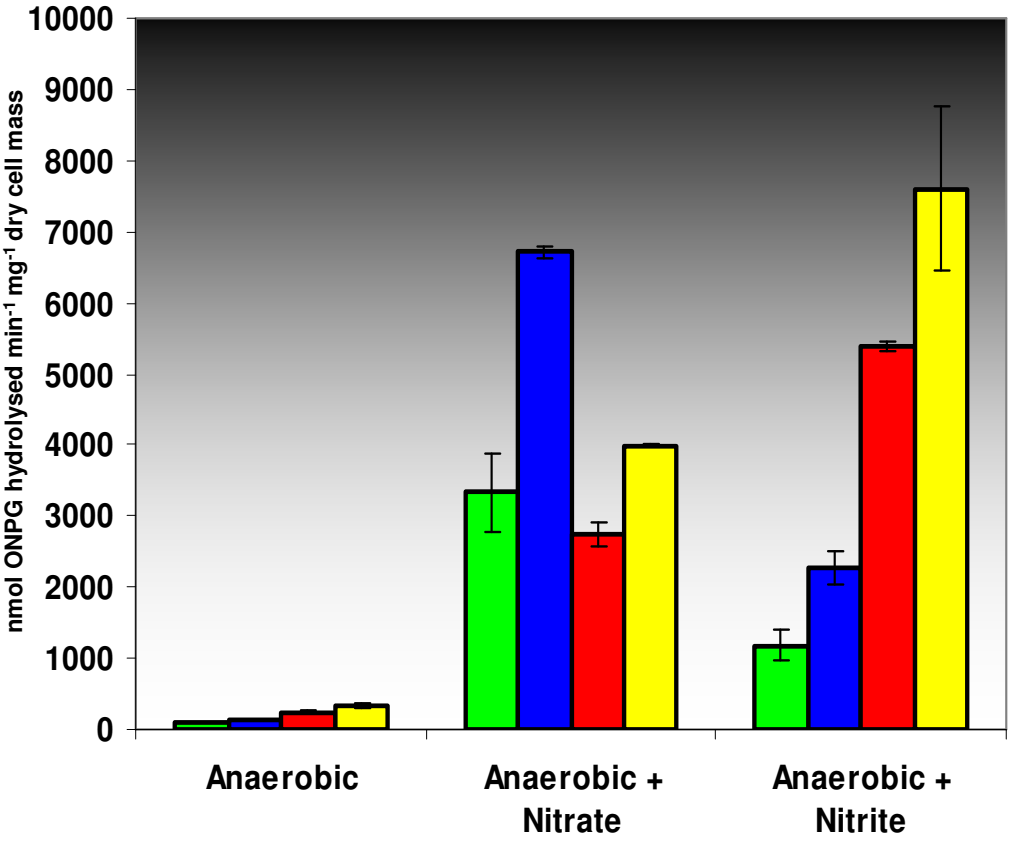

Figure 5.6 The effect of Fis on enhanced nitrite activation of *pyeaR* in an *fnr* mutant.

The *yeaR100* promoter fragment, cloned as a promoter-*lacZ* fusion in pRW50, was used to transform a parental *E. coli* strain, JCB387 (green) and strains mutated in the genes *fis* (blue), *fnr* (red) and *fis fnr* (yellow) respectively. Transformants were grown anaerobically in minimal medium in the presence or absence of nitrate (20 mM) or nitrite (2.5 mM), collected, lysed and assayed for promoter-driven β-galactosidase activity.

(blue) was higher than in the wild type in all conditions but highest promoter activity in the presence of nitrate was higher than in the presence of nitrite, following the expression profile of the wild type strain. This was not the case for the *fnr* null strain (red) that shows considerably higher activity in the presence of nitrite than in the presence of nitrate, which was a reversal of the normal promoter activity profile, a phenomenon previously described in this chapter as 'the FNR effect'. When both functional Fis and FNR were absent (yellow), the overall activity of the promoter in the presence of nitrite was further increased in comparison to the wild type and either single mutant, but the pattern of gene expression was similar to that of the *fnr* single mutant and the *fis* mutation appeared to have only an additive effect to the absolute promoter activity. Due to this evidence it was concluded that the increase in NarL dependent nitrite activation in the absence of FNR was not due to a secondary Fis effect.

Increased nitrite activation of the *yeaR* promoter in an *fnr*⁻ mutant is not due to increased cellular NarL levels

It has been established that the increased activity of the *yeaR* promoter in response to nitrite in an *fnr* mutant is not a direct effect of FNR binding to the promoter, nor a secondary effect of cellular Fis levels in strains with different growth rates. The simplest remaining explanation is that the absolute levels of NarL in an *fnr* null mutant are increased, thereby leading to an increase in all NarL-dependent, FNR-independent promoter activity. Published evidence on the regulation of the two-component regulator systems, NarXL and NarQP, using promoter-*lacZ* fusions has shown that although maximal expression of the unlinked genes, *narQ* and *narP*, requires anaerobiosis, activation of these genes was FNR and ArcA independent. In the case of the operon *narXL*, an *fnr* mutation has no effect on the anaerobic expression of *narX* but did cause an increase in absolute levels observed in anaerobic conditions in the presence of nitrite. This presumably leads to an increase in the levels of expression of the downstream-linked gene NarL, whose expression is driven both from the

inducible *narXL* promoter and at a constitutive low level from the *narL* promoter situated inside the *narX* reading frame (Darwin and Stewart, 1995; Egan and Stewart, 1990).

A Western blot was designed to ascertain whether the absolute cellular levels of NarL protein are increased in an *fnr* mutant strain in the presence of nitrite. The wild type control strain, JCB387, and the *fnr* null derivative, JCB3911, were grown anaerobically in the presence of nitrite. Strains mutated in *narL*, *narLP,* and *narX* (upstream of p*narL*) were also included as a negative control, to confirm NarL anti-body specificity and to confirm that *narL* is still expressed from the constitutive *narL* promoter in a *narX* mutant respectively. All strains were grown to an OD_{650} nm of ~0.5, at which point they were harvested and lysed in cracking buffer (see materials and methods). Equal biomass of cell lysates were separated by SDS-PAGE and transferred to nitrocellulose for Western analysis with rabbit anti-NarL antibody (anti-NarL1916) and horseradish-peroxidase anti-rabbit IgG secondary antibody (fig5.7).

Results confirm that the anti-NarL antibody, 1916, is specific to NarL only and does not cross-react with the NarL homologue, NarP (lanes 1-3). Comparison of the intensity of lanes 1 and 5, the wild-type strain and *fnr* null derivative respectively, indicate that the absolute cellular level of NarL in the *fnr* mutant was considerably lower than that of the wild type strain when corrected for biomass. This experiment mirrors the findings of a more intensive tandem-study of cellular-NarL levels in different strain and conditions, using Western analysis, completed by Meng Xu of the University of Birmingham (PhD thesis, 2009). To date, the reason for the lower expression of NarL in the *fnr* null strain has not been explained. Taken together this evidence suggests that any effect of an *fnr* mutation on the activity of the *yeaR* and *ogt* promoters is not due to an increase in the cellular levels of NarL protein.

NarL-dependent nitrate and nitrite activation of the *yeaR* promoter requires either sensor kinase, NarX or NarQ

In wild-type *E. coli* cells, it is accepted that the dual-acting two component systems, NarXL and NarQP govern the response of the bacterium to the presence of nitrate or nitrite in

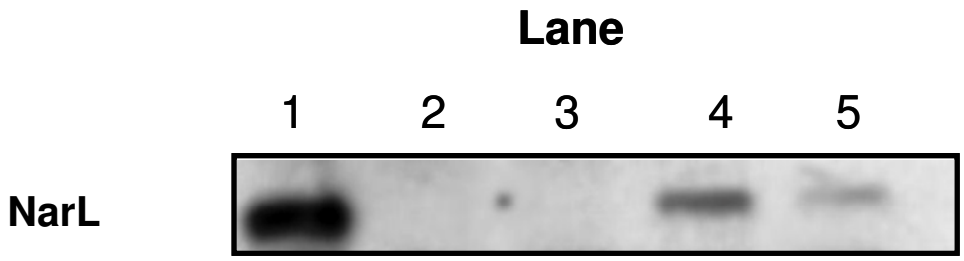

Figure 5.7 Western analysis of relative cellular quantities of NarL in mutant strains.

Samples 1, 2, 3, 4 and 5 were taken from strains JCB387 (parental strain), JCB3883 (*narL*), JCB3884 (*narLP*), JWK1213-1 (*narX*) and JCB3911 (*fnr*) respectively, grown in anaerobic conditions in the presence of 20 mM nitrate. Equal biomass of cell lysates were separated by SDS-PAGE and transferred to nitrocellulose for Western analysis with rabbit anti-NarL antibody and horseradish-peroxidase-labelled anti-rabbit IgG secondary antibody.

the environment. The fact that there is a significant difference in the sensitivity of sensor components, NarX or NarQ, to anions, nitrate or nitrite, and the fact that both response regulators, NarL and NarP can be phosphorylated by either sensor kinase means that there is a great deal of complexity in the control of nitrate and nitrite metabolism and significant cross talk between the systems. This is further complicated by the fact that in the presence of nitrite, NarX function reverses and NarL-phosphate is rapidly de-phosphorylated by NarX. Cross talk between the two-component systems has not only been observed in the case of NarXL and NarQP however, and some evidence has suggested histidine kinases (HK's) of unrelated two-component systems in the cell may cross-communicate with non-cognate response regulators (Yamamoto *et al.*, 2005).

To determine whether the NarL dependent activation of the *yeaR* promoter in response to nitrate or nitrite was dependent upon NarX, NarQ or another unidentified HK, a number of strains were constructed that were mutated to remove *narX, narQ* or *narXQ*. In all cases where the gene for *narX* had been disrupted, the expression of the downstream *narL* gene from p*narL* was confirmed by Western analysis using anti-NarL antibody (data not shown). The constructed strains, along with a wild type control, were transformed with the *yeaR100* promoter-*lacZ* fusion plasmid and grown anaerobically in the presence and absence of nitrate and nitrite. At mid-exponential growth phase the cells were harvested, lysed and assayed for β-galactosidase activity (fig 5.8). In the wild-type strain (red), the established expression profile was observed with high activity in the presence of nitrate and some increased activity in the presence of nitrite. Activity of the promoter in the absence of NarQ (blue) was unaffected in the conditions tested suggesting that NarQ is not required for the Nar-L-dependent activation of the *yeaR* promoter if NarX still functions. Similarly, nitrate induction of the *yeaR* promoter by nitrate in the *narX* mutant was unaffected (green), however, a reduction promoter activity in the presence of nitrite was observed in the *narX* mutant. This was especially surprising as established dogma would lead to the prediction that activity of a NarL-dependent promoter

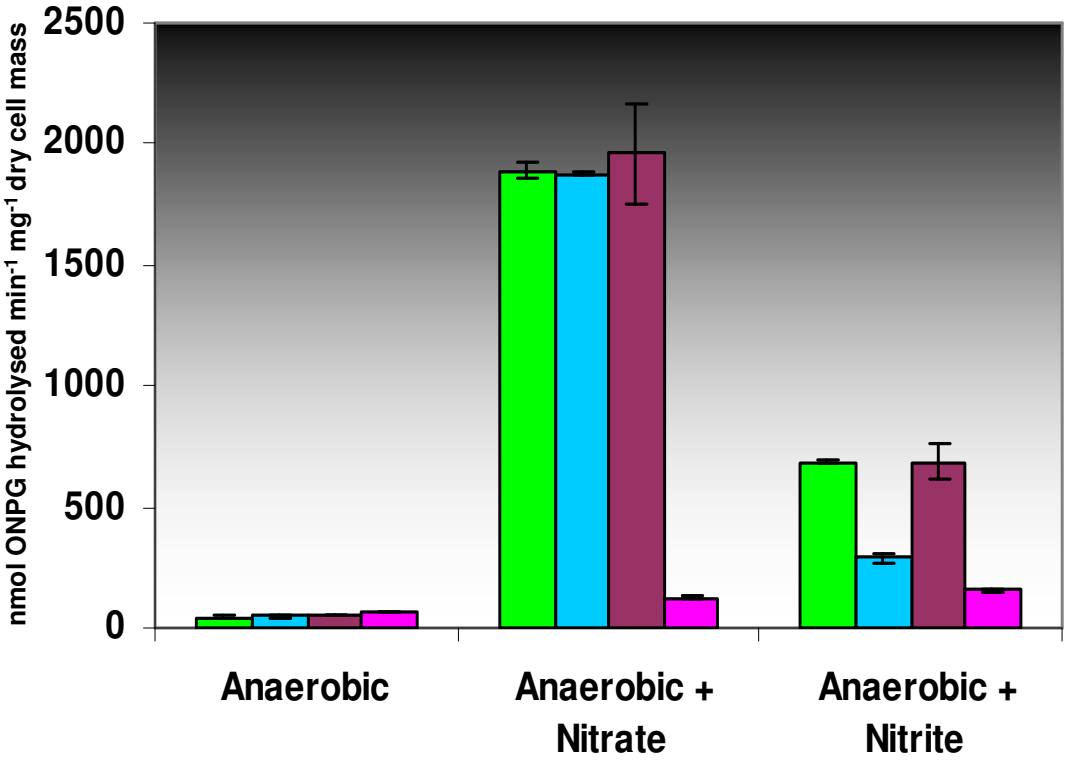

Figure 5.8 NarL dependent nitrate and nitrite activation of the *yeaR* promoter requires the sensor kinases NarX and NarQ

The *yeaR100* promoter fragment, cloned as a promoter-*lacZ* fusion in pRW50, was used to transform a parental *E. coli* strain, JCB387 (green) and strains mutated in the genes coding for *narX* (light blue), *narQ* (claret) and *narXQ* (pink) respectively. Transformants were grown anaerobically in minimal medium supplemented with nitrate or nitrite, harvested, lysed and assayed for β-galactosidase activity.

would be higher in the absence of NarX but the presence of NarQ when the strain is grown in nitrite, as NarX would normally dephosphorylate NarL under these conditions. Activity of the promoter in the *narXQ* double mutant (yellow) was un-inducible in all conditions tested proving that at least one of the sensor kinases, NarX or NarQ, wis required for activation of this promoter.

Increased NarL-dependent nitrite activation of the yeaR promoter in an fnr mutant is not due to FNR modulation of NarX phosphatase activity

The previous experiment demonstrated that the NarL-dependent activity of the *yeaR* promoter is dependent upon phosphorylation of NarL by either NarX or NarQ and that maximal promoter activity in the presence of nitrate was possible in the absence of NarX or NarQ indicating that both histidine kinases are able to sufficiently phosphorylate NarL in these conditions. As stated, one unique feature of the NarX protein is that in the presence of nitrite, NarX actively dephosphorylates NarL (Rabin and Stewart, 1993). The structural basis for the function of NarX in these conditions is not fully understood, however, one unique structural feature of the NarX protein sequence is the presence of an additional amino acid region in the central region of the protein, between the conserved Y-box and Q-linker (from His^{259} to Ile^{343} in NarX). This region is heterogeneous in both length and sequence to that of NarQ and contains a conspicuous pattern of conserved cysteine residues (Stewart, 2003)(Fig5.9). This led some to speculate that this region may co-ordinate an iron-sulphur centre that may influence the activity of NarX in response to oxygen. However later investigations have shown that in fact it is NarQ activity, which contains no conserved cysteine residues in this region, that is affected by culture aeration and it is the central region that determines this response (Stewart *et al.*, 2003). This study also confirmed, using sensor kinase chimeras, that it is the sensory domain or 'P-box' of NarX and NarQ that governs the differential response of the sensor kinases to nitrate and nitrite, not the central region. Although there is no established evidence for a direct protein-protein interaction between FNR and NarX and NarQ, the indirect effect of

Fig 5.9

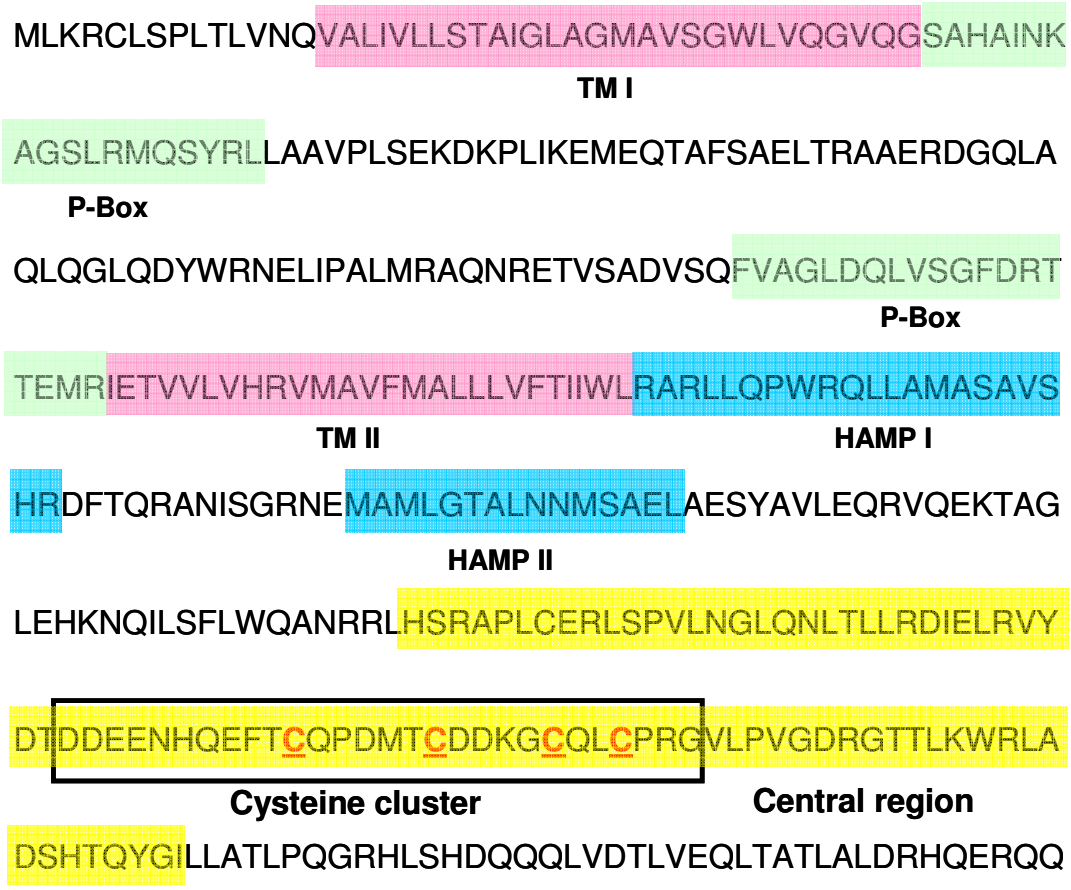
MLKRCLSPLTLVNQVALIVLLSTAIGLAGMAVSGWLVQGVQGSAHAINK
TM I
AGSLRMQSYRLLAAVPLSEKDKPLIKEMEQTAFSAELTRAAERDGQLA
P-Box
QLQGLQDYWRNELIPALMRAQNRETVSADVSQFVAGLDQLVSGFDRT
P-Box
TEMRIETVVLVHRVMAVFMALLLVFTIIWLRARLLQPWRQLLAMASAVS
TM II
HAMP I
HRDFTQRANISGRNEMAMLGTALNNMSAELAESYAVLEQRVQEKTAG
HAMP II
LEHKNQILSFLWQANRRLHSRAPLCERLSPVLNGLQNLTLLRDIELRVY
DTDDEENHQEFTCQPDMTCDDKGCQLCPRGVLPVGDRGTTLKWRLA
Cysteine cluster
Central region
DSHTQYGILLATLPQGRHLSHDQQQLVDTLVEQLTATLALDRHQERQQ
QLIVMEERATIARELHDSIAQSLSCMKMQVSCLQMQGDALPESSRELLS
QIRNELNASWAQLRELLTTFRLQLTEPGLRPALEASCEEYSAKFGFPVK
LDYQLPPRLVPSHQAIHLLQIAREALSNALKHSQASEVVVTVAQNDNQV
KLTVQDNGCGVPENAIRSNHYGMIIMRDRAQSLRGDCRVRRRESGGT
EVVVTFIPEKTFT DVQGDTHE

Fig 5.9 The domain organisation of the Sensor Kinase, NarX

This figure illustrates the NarX amino acid sequence with specific domains vital to its function highlighted. The approximate sequence of the two trans- membrane helices, TM I and TMII, the conserved P-Box, the HAMP-linker and the central region are highlighted in the purple, green, blue and yellow shaded boxes respectively. The additional sequence in the central region, which is unique to NarX, is highlighted in the boxed sequence and the conspicuous cysteine residues are shown in red underlined text.

FNR on nitrite activation of the *yeaR* and *ogt* promoters does indicate that there is some difference in signalling to NarL in an FNR mutant and, as shown previously (fig 5.8), all NarL dependent activation of the *yeaR*- promoter is NarX/ NarQ dependent. As in these conditions NarX would be expected to reduce NarL dependent activation through NarL-P phosphatase activity, it was postulated that FNR may directly modulate the phosphatase activity of NarX in response to nitrite, perhaps by interaction between cysteine rich regions present in both proteins.

To determine whether the absence of functional FNR has any effect on the signalling from NarX or NarQ, a number of mutants were constructed that had been disrupted in the genes *fnr narX, fnr narQ, and fnr narX narQ*. These strains, the parental strain, JCB387, and the *fnr* mutant strain, JCB3911, were transformed with the *yeaR100* promoter-*lacZ* fusion plasmid and grown anaerobically in the absence and presence of nitrate and nitrite. At mid-exponential phase the cells were harvested, lysed and assayed for β-galactosidase activity (fig 5.10). The activity of the wild-type strain (green) and *fnr* null mutant (red) followed established patterns, with the *fnr* mutant exhibiting increased promoter activity in response to nitrite. Promoter activity in the *fnr narX* mutant derivative (blue) was increased 1.6-fold in response to nitrate, 4-fold in response to nitrite and some increase in the basal level of the promoter was observed in the absence of nitrate or nitrite, compared to the parental strain. This suggested that NarX might indeed cause a reduction of phospho-NarL in an *fnr* null mutant. However, although overall activity of the promoter in a *fnr narX* background was higher, the same FNR effect of increase nitrite activation was observed in the *fnr*$^{-}$ *narQ* mutant derivative (yellow) indicating that the 'FNR effect' was not NarX dependent and NarQ-dependent NarL activation was also effected by the availability of FNR. Finally the activity of the promoter in the triple mutant, *fnr narZ narQ* was uninducible in any condition (mauve bars), proving that any effects of FNR on the activity of the *yeaR* promoter were NarXQ dependent.

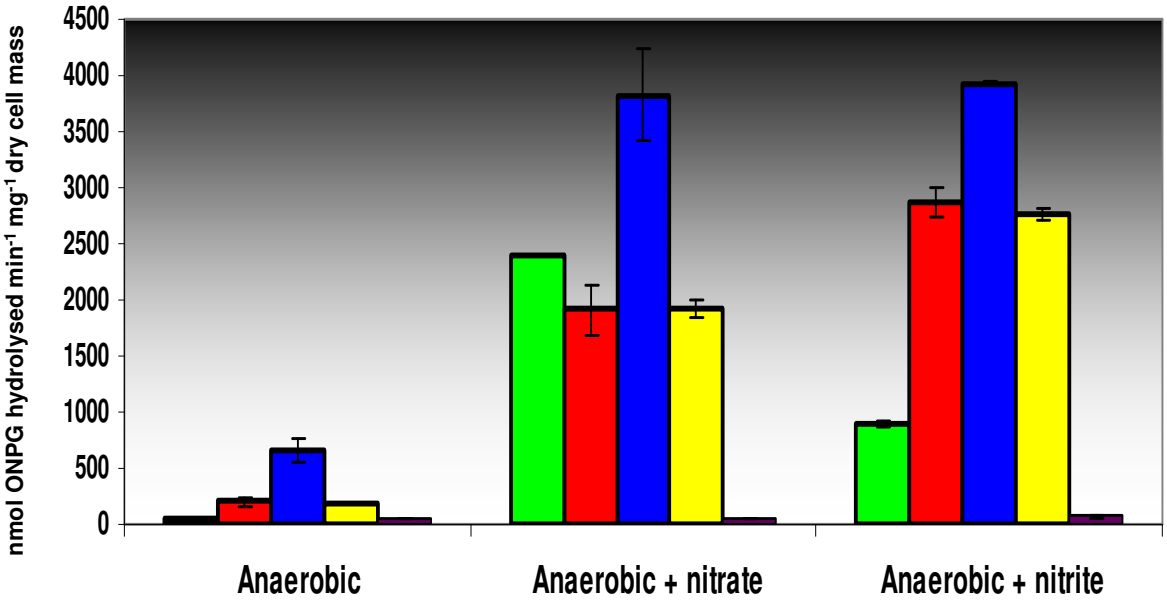

Figure 5.10 Increased NarL dependent nitrite activation of p*yeaR* in *fnr* null strains requires the sensor kinase NarX or NarQ.

Expression from the *yeaR100* promoter fragment, cloned as a promoter-*lacZ* fusion in pRW50, was measured in a parental *E. coli* strain, JCB387 (green) and strains mutated in the genes *fnr* (red), *fnr narX* (blue), *fnr narQ* (yellow) and *fnr narXQ* (mauve), respectively. Transformants were grown anaerobically in minimal medium in the presence or absence of nitrate (20 mM) or nitrite (2.5 mM). At mid-exponential growth phase cells were collected, lysed and assayed for promoter-driven β-galactosiade activity

These experiments have demonstrated that there is a complex relationship between FNR, NarX, NarQ and the apparent levels of NarL signalling in the cell that is not caused by the modulation of the normal function of the sensor kinases.

Identification of *yeaR*-promoter binding elements using DNA-sampling method

Investigation of the FNR transcriptome has revealed that the expression of many more genes than previously identified, including *yeaR-yoaG, ogt* and many other genes of unknown function, are regulated directly or indirectly by the action of FNR (Constantinidou *et al.*, 2006). These hypothetical proteins could include putative transcription factors or DNA binding proteins that have so far been unidentified. In order to determine whether any other factors, with known or unknown function, bind to the *yeaR* promoter region, a recently developed DNA-sampling technique was utilised (Butala *et al.*, 2009). This method allows for rapid isolation of specific DNA fragments together with attached proteins, directly from *E. coli* K-12 cells grown in any condition. The *yeaR100 Eco*RI-*Hin*dIII promoter fragment was ligated into a low-copy number plasmid, pWR901, at a site adjacent to multiple operator binding sites (LacO) for the LacI repressor and between two sites for the yeast Sce-I meganuclease. This plasmid, pDJS902 was co-transformed with the multi-copy plasmid, pACBSR-DL1 into the strains, JCB387Δ*lacI::*FLAG and the *fnr* null derivative, JCB3911Δ*lacI::*FLAG, by electroporation The multi-copy plasmid pACBSR-DL1 is a derivative of pACBSR (Scarab genomics), which encodes the modified lambda *red* system (encoding only the Gam protein) and the I-SceI meganuclease under arabinose inducible promoters (Butala *et al.*, 2009). This method allows for the isolation of promoter DNA-protein complexes by ustilising affinity isolation using anti-FLAG antibodies directed towards FLAG-tagged LacI, which will bind upstream of the promoter fragment of interest at the multiple Lac operator sites (fig. 5.11). Transformants were grown anaerobically to mid-exponential growth phase in the presence of

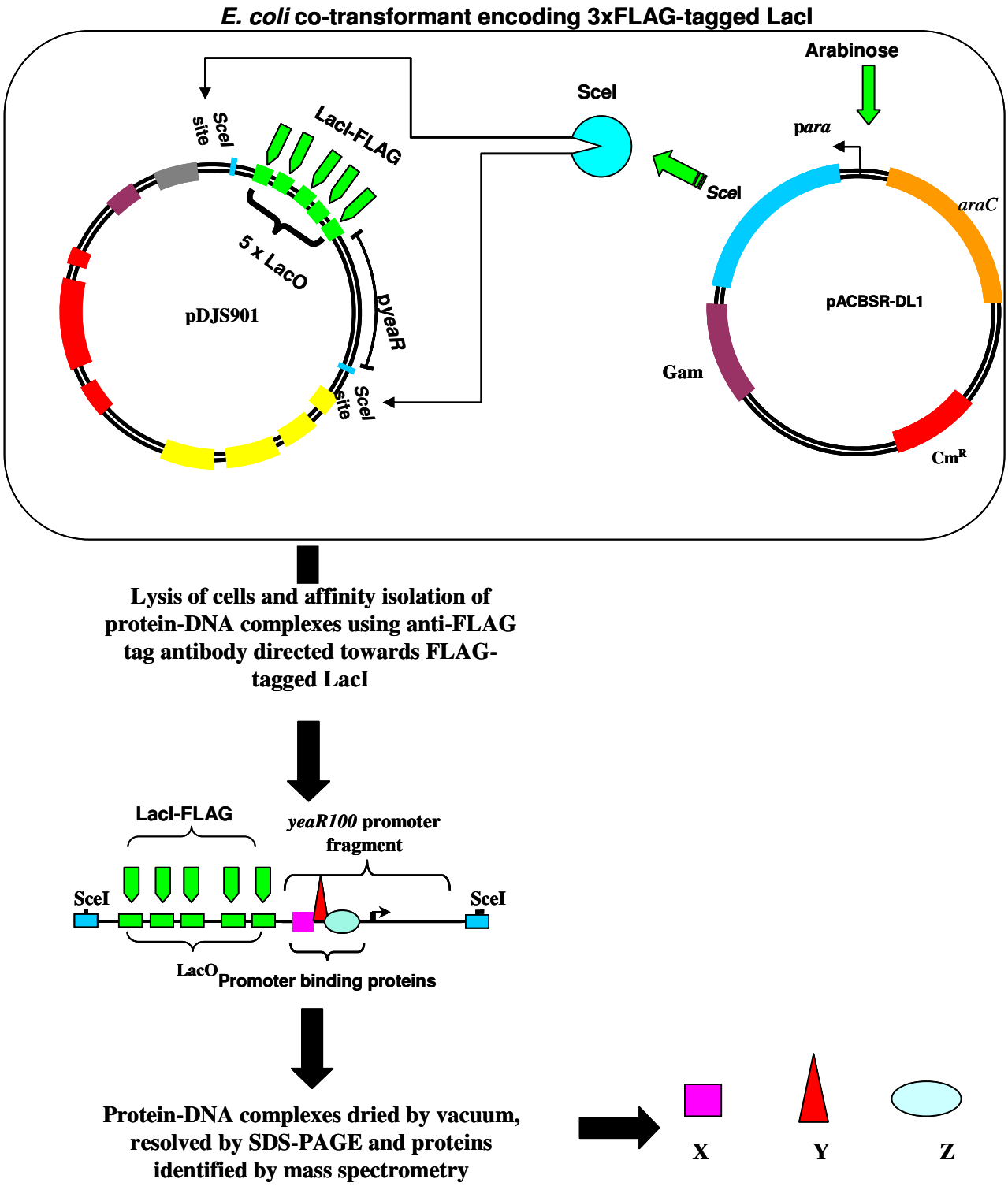

Figure 5.11 Outline of the DNA-sampling method for probing protein binding at bacterial promoters described in materials and methods and by Butala *et al.*, (2009).

nitrite. At this point cells were treated with 0.4% arabinose, which induces the expression of SceI from pACBSR-DL1. The action of SceI then excises protein-DNA complex, consiting of the linear promoter fragment, bound at the *lac* operators by LacI-FLAG and any promoter bound proteins. After lysis, protein-DNA complexes are isolated by the affinity isolation method described in materials and methods, resolved by SDS-PAGE before bands of interest are extracted, digested with trypsin and identified by mass spectrometry.

Comparison of the protein-band profile of the wild-type and *fnr* mutant strain identified at least seven candidate proteins that were either unique to particular strains or present in significantly different quantities in each sample (fig 5.12). These bands were excised, destained, digested with trypsin and resultant peptides were extracted and analysed on a Thermo-Finnigan FT-ICR mass spectrometer using a NanoMate chip-based electrospray system operated by the University of Birmingham Functional Genomics and Proteomics Unit. Candidate proteins 1-3 were identified as nucleoid associated proteins, H-NS, IHF and HU respectively. As IHF was known to bind to the extreme upstream region of the *yeaR100* fragment, outside of the promoter region (data not shown), this candidate was disregarded. The effects of H-NS and HU on activity of the *yeaR* promoter have not been determined. All other candidate bands were found to contain degradation products of LacI-FLAG and were also disregarded.

From this experiment it did not appear that an unidentified transcription factor binds to the *yeaR* promoter in the presence or absence of FNR and repress or enhance activity accordingly.

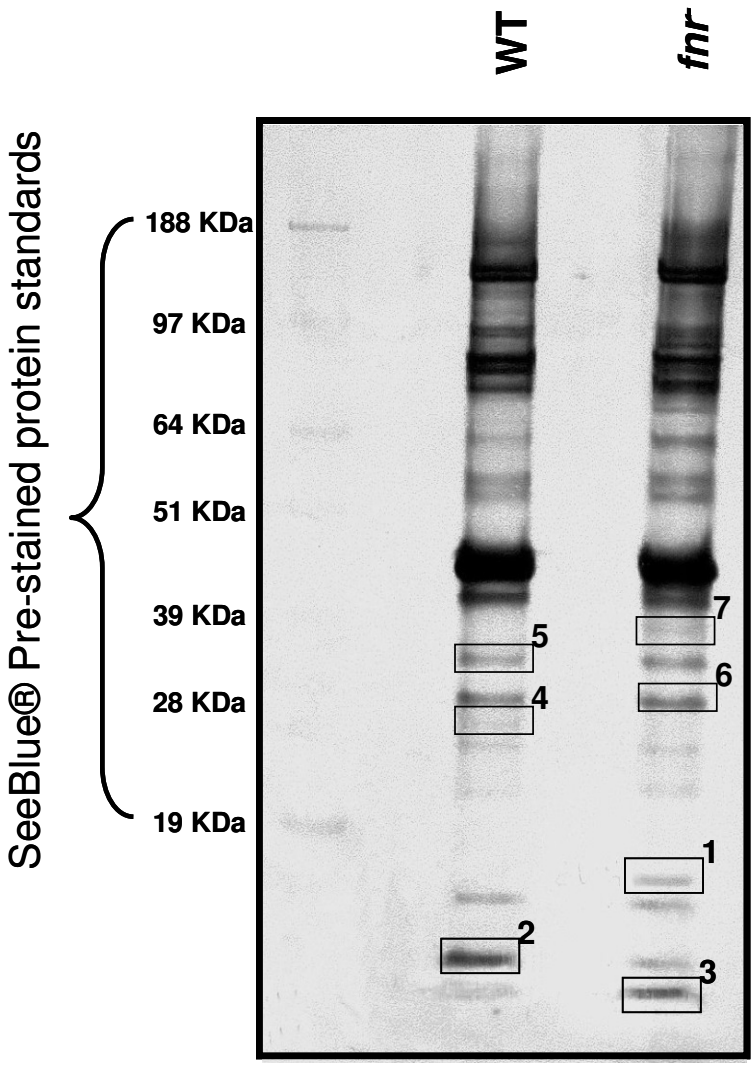

Fig 5.12 Isolation of *yeaR*-promoter binding proteins using the DNA-sampling method

Strains JCB387*lacI-flag* and JCB3911*lacI-flag* were co-transformed with the plasmids pYEAR901 and pACBSR-DL1, grown anaerobically to an OD_{650} of 0.5 in the presence of 2.5 mM nitrite and induced with 0.4% arabinose. Cells were harvested, lysed by sonication and pure extract was obtained. Promoter DNA-protein complexes were isolated by incubation with anti-FLAG conjugated magnetic beads, washed and eluted. Proteins were separated by SDS-PAGE on a 4-12% gradient gel, calibrated for size with SeeBlue® prestained protein markers.

Protein bands observed in different abundance when samples were compared were extracted, de-stained, digested with trypsin and analysed on a Thermo-Finnigan FT-ICR mass spectrometer using a NanoMate chip-based electrospray system.

Chapter 6

Discussion

The regulation of NarL dependent promoters

Transcription activation by NarL in *Escherichia coli*

Transcription activation in *E. coli* often requires the binding of transcription factors proximal to the RNA polymerase binding site and protein-protein contact between the transcription factor and RNA polymerase. Transcription activators can function in a class I, class II or class III manner, either by binding upstream of the α-CTD binding site and making a single contact with the RNAP enzyme (class I), binding between the -35 promoter element and the α-CTD binding site and contacting two or more elements of the RNAP holo-enzyme (class II), or in a combinations of these two arrangemnents (class III) (Browning and Busby, 2004). The global transcription activator, FNR, can function as both a class I and a class II activator (Li *et al.*, 1998). At many FNR dependent promoters, the transcription activators, NarL and NarP, also bind to the promoter region and enhance promoter activity (Stewart, 1993). However, at the start of this project, no promoter had been shown to be activated by NarL independently of FNR. Data generated during this study and in a parallel independent study (Lin *et al.*, 2007) have shown that the expression of the operon of unknown function, *yeaR-yoaG*, is dependent only on NarL for transcription activation both *in vivo* and *in vitro*. Measurements of transcription activation *in vivo* revealed that *yeaR* promoter activity is totally dependent upon NarL (table 4.1). The activation of p*yeaR* by NarL *in vivo* was also shown to be independent of FNR, suggesting that NarL is capable of activating transcription without the need for co-activation by FNR (fig. 4.7).

Investigation of the *in vitro* binding of NarL to the *yeaR-yoaG* promoter has revealed that NarL binds to a site centred around 47.5 bp upstream of the transcription start site (fig 4.5),

and leads to an approximately two-fold increase in promoter opening by RNAP *in vitro* (fig. 4.10). The total dependence upon NarL for *in vivo* activation of *yeaR-yoaG* and the binding position of NarL at the promoter strongly suggest that NarL is able to function independently as a class II transcription activator at this promoter.

In a parallel study by Meng Xu (University of Birmingham), the activity of the *ogt* promoter was also shown to be dependent upon NarL for activation and is independent of FNR. *In vitro* investigation revealed that the *ogt* promoter region contained two binding sites for NarL of equal affinity, centred at positions -45.5 and -78.5 with respect to the transcription start site (Squire *et al.*, 2009). Mutagenesis revealed that activity of the promoter is dependent upon NarL binding to both of these operators, although co-operative binding was not ruled out. The dependence upon both NarL sites for promoter activity and the positioning of the NarL operator sites relative to the transcription start site suggest that the *ogt* promoter is activated by NarL via a class III mechanism.

The ability of NarL to independently activate transcription by both a class II and class III mechanism at multiple promoters shows that not only is NarL a competent transcription activator, but that it also demonstrates versatility in the mechanisms of transcription activation (fig 6.1).

Future investigation: the activating regions of NarL

When FNR functions as a class I activator it binds to a site, typically centred at position -61.5, and makes a single contact with the α-CTD of RNA polymerase at a specific region known as activating region 1. At promoters where FNR activates via a class II mechanism, FNR binds to a site centred at position -41.5 and makes contact with the RNAP holo-enzyme at three specific regions: Activating region 1 contacts the upstream bound α-CTD of RNAP; activating region 2 contacts the α-NTD of RNAP; and activating region 3 contacts domain 4 of sigma factor of the RNAP holo-enzyme (fig.1.7). The activating regions of FNR have been

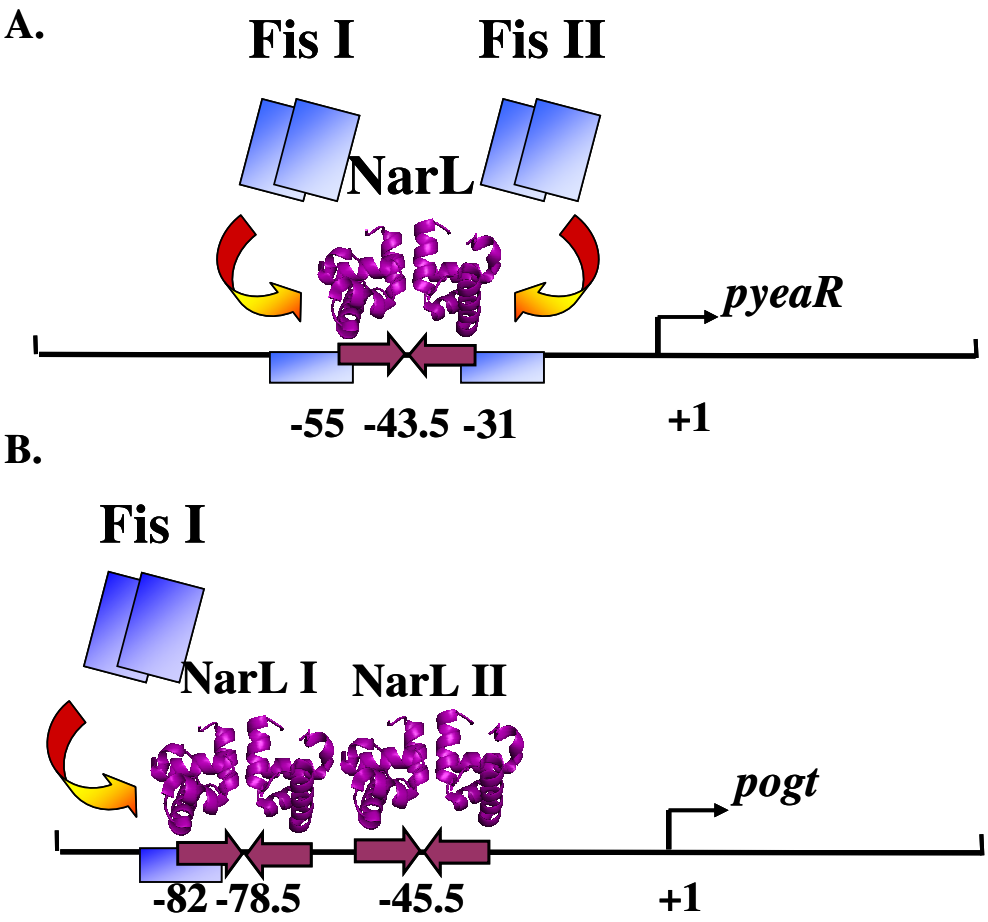

Figure 6.1 Proposed mechanism for transcription regulation of the NarL-dependent promoters, p*yeaR* and p*ogt*

A. At the *yeaR* promoter, NarL (purple) binds to a single 7-2-7 inverted repeat operator centred at -43.5 and activates transcription, presumably by a class II mechanism. Fis (blue boxes) represses *yeaR* expression by binding at two sites that overlap the NarL operator and occlude binding by NarL.

B. At the *ogt* promoter, NarL (purple) binding to two 7-2-7 inverted repeat sites is required for transcription activation. NarL is presumed to make several contacts with different subunits of RNAP and activate transcription by a class III mechanism. Fis (blue boxes) also represses the *ogt* promoter by binding to a single site that overlaps the upstream NarL site, NarL I, occluding NarL binding. Both the *ogt* and *yeaR* promoters share similar regulatory features.

shown to be essential for both classes of transcription activation by mutating specific amino acids within these regions (Li *et al.*, 1998). Due to the fact that NarL has never been shown to function as an independent transcription activator, how or where NarL contacts RNA polymerase is unknown.

An investigation to identify the activating regions of NarL would be necessary for understanding how NarL is able to function as a transcription activator. One method for identifying the specific regions of the NarL protein that are essential for activation comes from the work of Lee *et al* (2000). This study identified the essential residues of RNAP required for interaction with FNR at class I promoters by utilising suppression genetics and an existing alanine-scan library of the αCTD domain of RNA polymerase (Murakami *et al.*, 1996). By determining the 'patches' of residues in RNAP that are essential for activation by FNR, the residues that interact during transcription activation could be identified. By using p*yeaR* as a reporter of NarL-dependent transcription activation and the α-CTD alanine scan method, the residues of the RNA polymerase α-CTD that are essential for activation of p*yeaR* by NarL could be identified and mapped onto the experimentally determined structure of the α-CTD. From this information it is likely that a number of essential 'patches' will be identified. By combining information on the properties of the essential amino acids in the α-CTD, such as charge and hydrophobicity, with the determined structure of NarL, it might be possible to identify candidate residues in the structure of NarL that can then be targeted for mutagenesis. This should lead to the discovery of how and where NarL contacts RNA polymerase at the *yeaR* and *ogt* promoters in order to activate transcription. The structure of the DNA-binding C-terminal domain of NarL bound to oligonucleotides containing NarL operators from the *nir* and *nar* promoter DNA sequences has been resolved (fig 6.2) (Maris *et al.*, 2005). This information could also aid in the understanding of how NarL functions as a transcription activator.

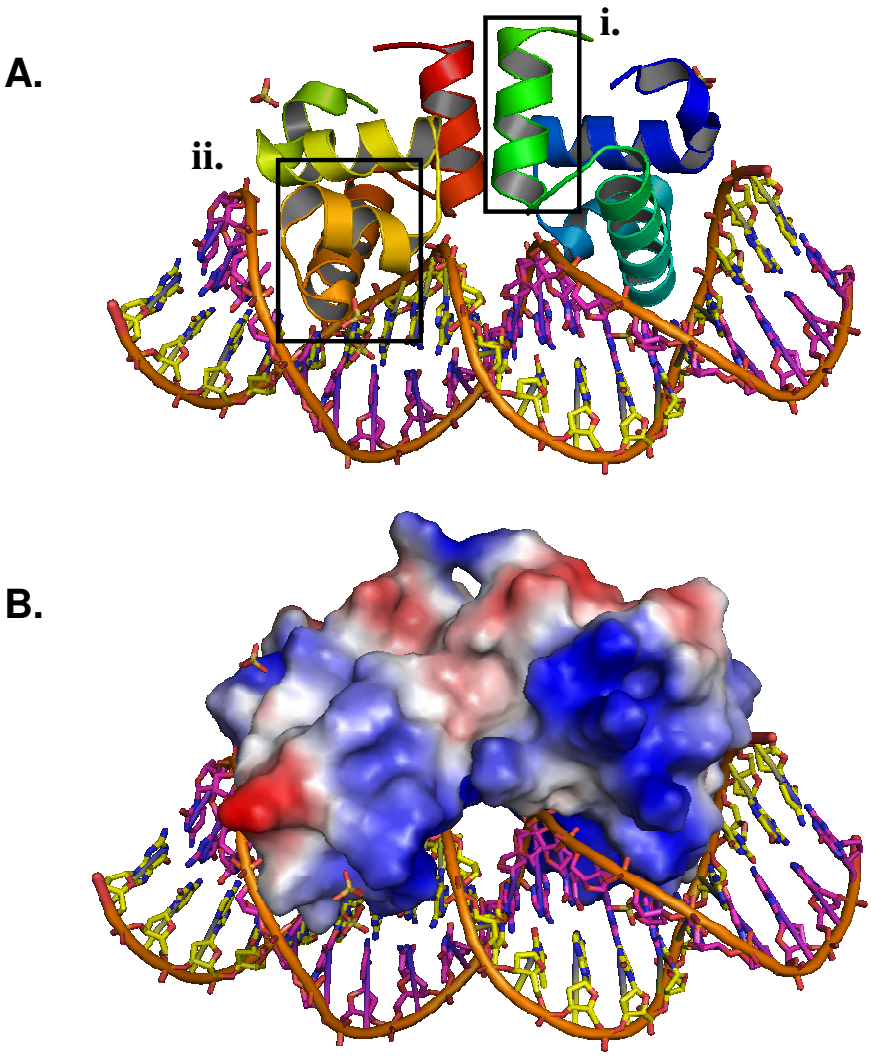

Fig 6.2 X-ray crystal structure of NarL-CTD DNA binding domain complexed with oligonucleotides containing NarL target sequences.

A. Co-crystallisation of the NarL C-terminal domain with DNA oligonucleotides containing NarL binding sequences revealed that dimerisation of NarL at the *nirB* promoter occurred independently of the N-terminal sensor domain (accession number 1ZG1)(Maris *et al*., 2005). The dimeristaion occurs via the C-terminus helix (**box i.**) while the DNA recognition helix facilitates sequence recognition **(box ii**.).

B. Predicted positively (blue) and negatively (red) charged regions of the NarL-CTD protein dimer when complexed with DNA. These exposed charged regions may be important in protein-protein interactions with the RNAP α-CTD.

NarL binding site positioning and promoter strength

In contrast to the *yeaR* promoter, which requires binding of NarL to only one site for transcription activation, activation at the *ogt* promoter requires binding of NarL to two sites in the promoter region. One explanation for this is the fact that binding of NarL to the *ogt* promoter has been shown to be ~10 times weaker than binding at the *yeaR* promoter *in vitro* (Squire *et al*., 2009). This could account for the difference in overall promoter activity in response to nitrate and the lack of nitrite induction of *ogt* when phospho-NarL levels would be lower. Furthermore, the positioning of the *yeaR* NarL binding site at -43.5 is 2 bp closer to the -35 promoter element than is the downstream NarL binding site at *ogt*, which is centred around -45.5. It was initially postulated that the change in positioning of the downstream NarL operator at p*ogt* might affect the strength of the *ogt* promoter. This might explain the requirement for a second upstream NarL binding site at the class III activated *ogt* promoter in comparison to the class II NarL-dependent *yeaR* promoter. The requirement for two sites in the *ogt* promoter has been demonstrated (fig 4.15). However, to date, it has not been demonstrated whether the binding of NarL to the two sites in the *ogt* promoter is co-operative and whether mutagenesis of one site leads to a loss of binding at both sites. To determine whether this is the case, a DNase I footprint with the two mutagenised *ogt* promoter fragments OGT102 and OGT104 could be used. If NarL still binds to one site when the other site has been mutagenised, it will prove that NarL binding at each site is independent and suggest that NarL makes multiple contacts with RNAP at the *ogt* promoter. However, if loss of binding at one mutagenised site causes loss of all binding of NarL to the *ogt* promoter, this would suggest co-operative binding of NarL and would require further investigation.

Initial investigations by David Chismon (unpublished results) into the properties of the NarL operator sequence and positioning in the *yeaR* and *ogt* promoter regions yielded some unexpected results. When the NarL operator from the *yeaR* promoter was transplanted into the *ogt* promoter to replace the *ogt* NarL operator (-45.5), *ogt* promoter activity was increased to

approximately the same level as the *yeaR* promoter in response to nitrate. Conversely, when 2 bp of promoter DNA between the -35 promoter element and the downstream *ogt* NarL operator was deleted, to reposition the *ogt* NarL-operator in the *yeaR* promoter position (-43.5), it led to a total loss of *ogt* promoter activity in response to nitrate. This evidence suggests that it is the sequence of the *yeaR* NarL operator, and therefore the binding affinity of NarL, that governs the overall activity of the promoter, and not the position of the operator relative to the -35 and -10 promoter elements. However, this experiment also reveals that the exact spacing of the NarL operator with respect to the *ogt* transcription start site is vital to promoter function.

The NarP independence of *yeaR* promoter activity

As has been demonstrated in this study and previous investigations, the binding sites for NarL at the *yeaR* and *ogt* promoters are arranged as 7-2-7 inverted repeat sequences (Lin *et al.*, 2007; Squire *et al.*, 2009). This arrangement is usually sufficient for binding of both NarL and the homologous response regulator, NarP (Darwin *et al.*, 1997; Tyson *et al.*, 1994). However, thorough investigation of both p*yeaR* and p*ogt* has shown that NarP has little or no effect at either of these promoters: NarP does not bind to either promoter *in vitro* (fig 4.6) and very little effect on promoter activity is seen *in vivo* (table 4.1). This phenomenon is not believed to be due to a difference in affinity for the operator sequences, as even at high concentrations of NarP, no binding was observed in EMSA experiments with the *yeaR* promoter fragment.

The NarL and NarP amino acid sequences ar 44% identical, 64% similar and differ in length by a single amino acid (fig 6.3). Although the structure of NarP has not been solved, based on its amino acid sequence it is presumed to have a similar three-dimensional structure to that of NarL. The amino acid sequences of NarL and NarP are most similar in the sensor domains and the DNA binding helix-turn-helix domains, but differences in other regions could account for the inability of NarP to function as an FNR-independent transcription activator.

Figure 6.3

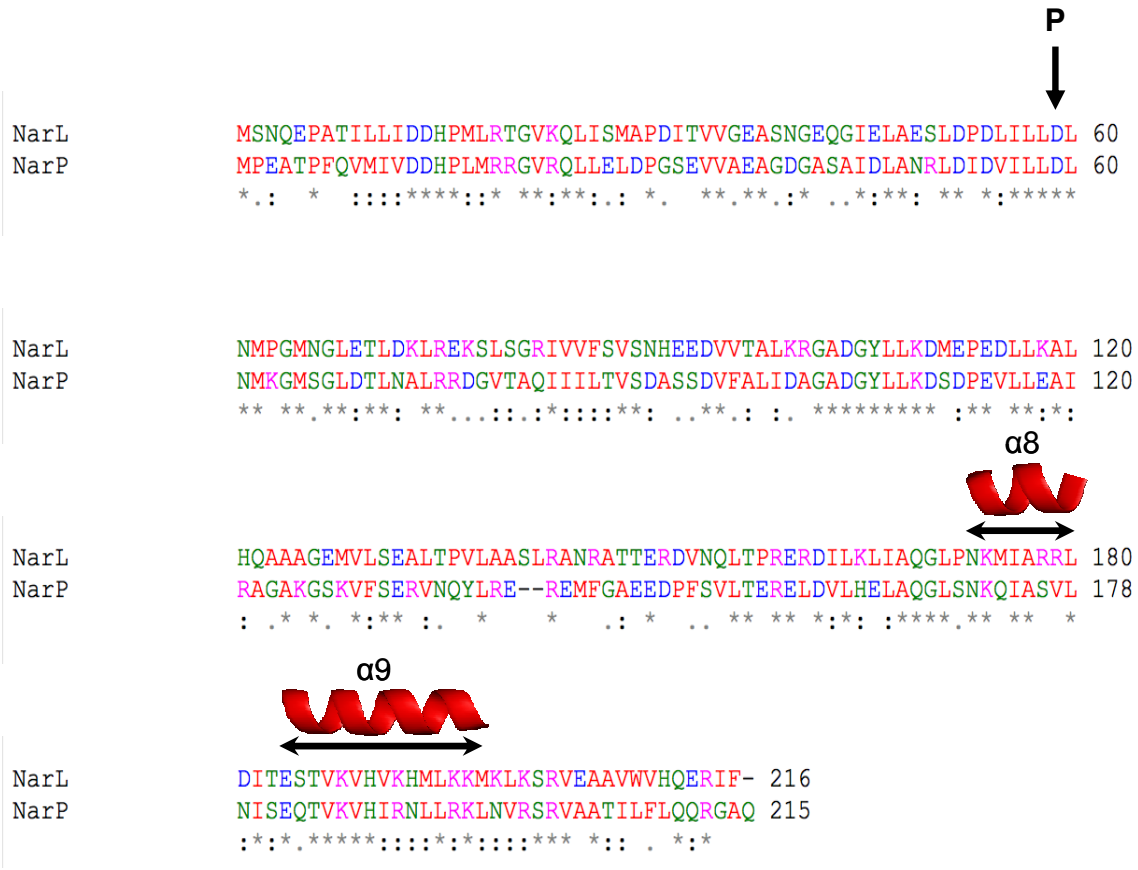

Figure 6.3 Alignment of the amino acid sequences of the homologous response regulators, NarL and NarP

The amino acid sequences of the homologous response regulator proteins, NarL and NarP, were aligned using the ClustalW tool at http://www.ebi.ac.uk/Tools/clustalw2. Residues that are identical in the two proteins are marked with a star, while residues with side chains with very similar or related properties are marked with a colon or full stop, respectively. The helix-turn-helix DNA binding motif, formed by helices α8 and α9, and the conserved apsartate (the site for phosphorylation) are indicated.

The physiological relevance of *yeaR* and *ogt* regulation by NarL but not NarP is not fully understood, especially when the effect of an *fnr* mutation on increased NarL dependent nitrite activation is considered.

The role of NsrR at the *yeaR* promoter

The expression of *yeaR* has been shown in various transcriptomic studies to be increased in response to nitric oxide and other reactive nitrogen species (see table 1.1). This response is largely attributed to the presence of an NsrR binding site in the *yeaR* promoter region that overlaps the -35 promoter element. An investigation into the NsrR regulon showed a 4-fold increase in the transcription of *yeaR* in conditions where NsrR repression had been relieved by repressor titration (Filenko *et al.*, 2007). However, during this study, the repression effect of NsrR on p*yeaR* has been found to be weak (fig 4.7). It is difficult to determine the extent of NsrR repression at the *yeaR* promoter using *in vivo* promoter-*lacZ* fusion assays due to the requirement for nitrate activation via NarL. When nitrate is present, NarL activates transcription from the *yeaR* promoter and, presumably, NsrR represses until nitric oxide is produced. However, nitric oxide is already presumably being produced by the bacterium as a result of nitrate and nitrite reduction, meaning that NsrR is already de-repressed. Indeed, the introduction of an *nsrR* mutation has very little effect on the overall promoter activity in these conditions, suggesting that promoter activity is maximal and NsrR is already de-repressed.

In order to elucidate what role the NsrR protein has in regulation of the *yeaR* promoter, it would be necessary to use both *in vivo* and *in vitro* techniques. Some success using crude cell lysates of strains over expressing functional NsrR for EMSA experiments has been recorded by Doug Browning and David Chismon (University of Birmingham). These experiments have shown that the promoter fragments of *nrf* and *hcp*, which both contain NsrR operators, are bound by a protein, presumed to be NsrR, changing the electromobility of the promoter

fragment (unpublished data). This approach could be used to ascertain whether NsrR does bind to the *yeaR* promoter.

More recent evidence has shown that it is possible to purify NsrR from other bacterial species and to use the purified protein in EMSA experiments (Tucker *et al.*, 2008). If this approach can be used in *E. coli,* it would be more desirable to use purified protein than crude cell extract. However, all purifications and subsequent reactions using the NsrR protein must be carried out in an anaerobic cabinet.

A further method for determining the role of NsrR in the regulation of *yeaR* would be the use of 'star' or 'locked on' mutants of NarL. If the activity of NarL is made independent of the presence of nitrate or nitrite, the activity of the *yeaR* promoter could be assayed in the absence of nitrate in strains with and without functional NsrR. This would allow the effect of NsrR to be measured in the absence of any source of reactive nitrogen species, while NarL is still fully active. Although a 'locked on' NarL mutant is not currently available, it has been shown previoulsy that if the NarQ sensor kinase from *Neisseria gonorrhoeae* is introduced into *E. coli*, it continually phosphorylates *E. coli* NarL even in the absence of nitrate or nitrite (Overton *et al.*, 2006; Whitehead and Cole, 2006). This would provide a suitable alternative method for testing the role of NsrR at the *yeaR* promoter in the absence of sources of nitric oxide.

Aerobic inhibition of the *yeaR* promoter

Once it was established that the activity of the *yeaR* promoter is activated by NarL in the presence of nitrate, independently of FNR, it was presumed that the activity of the promoter would be induced equally in the presence of nitrate under aerobic growth conditions. Initial experiments demonstrated that this was not the case, and that the activity of the promoter under aerobic conditions was approximately 10-fold lower than the absolute level observed in anaerobic conditions (fig. 4.7, 4.12, 4.13 and 4.14). The initial explanation for this

phenomenon was that, in aerobic conditions, the nitrate and nitrite reductases, whose expression is FNR dependent, are not synthesised. This would mean that there would be no endogenous source of nitric oxide in the cells during aerobic growth to relieve repression by NsrR, leading to low promoter activity. However, this was shown to be incorrect, as the introduction of a chromosomal *nsrR* mutation had very little effect and the promoter activity remained low. This result is contradictory to the findings of a parallel study on the regulation of *yeaR*, which showed a similar pattern of aerobic inhibition but greater overall promoter activity in aerated cultures in the presence of nitrate (Lin *et al.*, 2007). Lin *et al* (2007) also showed that by mutagenising the NsrR operator in the *yeaR* promoter they were able to drastically increase promoter activity in both aerated and non-aerated cultures.

The reason for this anomalous result is not clear, but it could be due to differences in the methods used in this study and that of Lin *et al* (2007). First, all promoter-*lacZ* fusions in this study were constructed in the low-copy number plasmid (2-5 copies per cell), pRW50, whereas Lin *et al* used single-copy chromosomal promoter-*lacZ* fusions at the bacteriophage λ insertion site. The difference in the overall effect of NsrR at the *yeaR* promoter between the two studies could also be due to the use of plasmid-based promoter-*lacZ* fusions, as NsrR has been shown to be prone to repressor titration (Bodenmiller and Spiro, 2006; Filenko *et al.*, 2007). This could mean that the *yeaR* promoter is already partially de-repressed with regards to NsrR, in all experiments in this study, due to the presence of multiple NsrR operators and repressor titration.

NarX dependent NarL phosphorylation in response to nitrite

The dependence of NarL-activation at p*yeaR* on the sensor kinases, NarX and NarQ, was determined during this project (fig 5.8). It was determined that either NarX or NarQ was required for full activation of the promoter by NarL. However, a surprising anomaly was observed when the activity of the *yeaR* promoter was measured in a *narX* null derivative in the

presence of nitrite. Existing evidence, outlined in chapter five, would suggest that under these conditions, the activity of a NarL dependent promoter would be higher than in the parental strain where NarX is functional. This would be because of the antagonistic action of NarX phosphatase activity in the presence of nitrite, which would limit the cellular levels of NarL, phosphorylated by NarQ (Rabin and Stewart, 1993). When NarX is absent, it would be expected that NarQ would phosphorylate NarL unimpeded by the action of NarX, leading to an increase in NarL-dependent promoter activity. This was clearly not the case in this study and remains unexplained but represents a further aspect of interest in the anaerobic nitrate respiration regulatory network.

The action of Fis at NarL-dependent promoters

The activity of both the *yeaR* and *ogt* promoters was similarly repressed when assayed in rich medium, and this effect was shown to be due to the nucleoid associated protein, Fis (figs 4.8 & 4.16). Fis was shown to bind to two sites in the *yeaR* promoter and a single site in the *ogt* promoter. Remarkably, at both promoters it appeared that Fis was able to repress transcription by binding to one or more sites that overlapped the NarL operator, effectively excluding an essential activator.

It is not entirely clear as to why the cell would opt out of expressing *yeaR* and *ogt* in times of rapid growth in rich medium, conditions when Fis levels would be high. However, it is well established that *E. coli* represses the transcription of many essential genes during rapid growth due to the limited supply of RNA polymerase, which is diverted to the promoters of genes encoding the protein synthesis and cell replication machinery (Ishihama, 2000). In several cases, this repression effect is facilitated by Fis by various different mechanisms (Browning *et al.*, 2004a; Browning *et al.*, 2005; Grainger *et al.*, 2008). This study provides further evidence of the diversity of roles of the nucleoid associated protein, Fis.

Indirect repression of NarL dependent promoters by FNR in response to nitrite

Increased levels of NarL phosphate in the absence of functional FNR

The activity of the NarL-dependent *yeaR* promoter was shown, in this investigation, to be increased relative to the parental strain in response to nitrite in the absence of functional FNR (fig 5.4). This result, as well as results of previous micro-array studies, suggested that FNR may act as a repressor of this promoter (Constantinidou *et al.*, 2006). However, *in vitro* investigation showed that purified FNR protein does not bind specifically to the *yeaR* promoter region, meaning that any observed effect on promoter activity must be via an indirect mechanism. Western analysis of the cellular levels of NarL showed that the increase in *yeaR* promoter activity in an *fnr* mutant was not due to an increase in total cellular NarL content, which was in fact lower in an *fnr* null derivative (fig 5.7). Chapter 5 of this study investigated this phenomenon in detail and established that the effect of an *fnr* mutation was not due to modulation of NarX phosphatase activity, as NarQ-dependent NarL activity was also shown to be affected by an *fnr* mutation (fig 5.10). Furthermore, the apparent 'FNR effect' was observed in the case of the *ogt* promoter, indicating that this is a general effect of FNR on NarL-dependent promoters (fig 5.4).

Taking into account all of the evidence gathered during this investigation, it was postulated that, by some unidentified mechanism, there is an increase in the cellular levels of NarL-phosphate in response to nitrite in an *fnr* mutant strain. In order to test this hypothesis, an experiment was attempted to determine the ratio of NarL-phosphate: total NarL, using highly sensitive mass spectroscopy. This method would require the identification of the peptide containing the phosphorylation site Asp59 (fig 6.4). A strain was constructed, in which the chromosomal *narL* gene was replaced with a gene encoding a C-terminal 3xFLAG fusion of NarL. This strain was grown anaerobically in the presence of nitrite and total cellular NarL-FLAG was extracted using anti-FLAG antibodies conjugated to magnetic beads. This

Figure 6.4

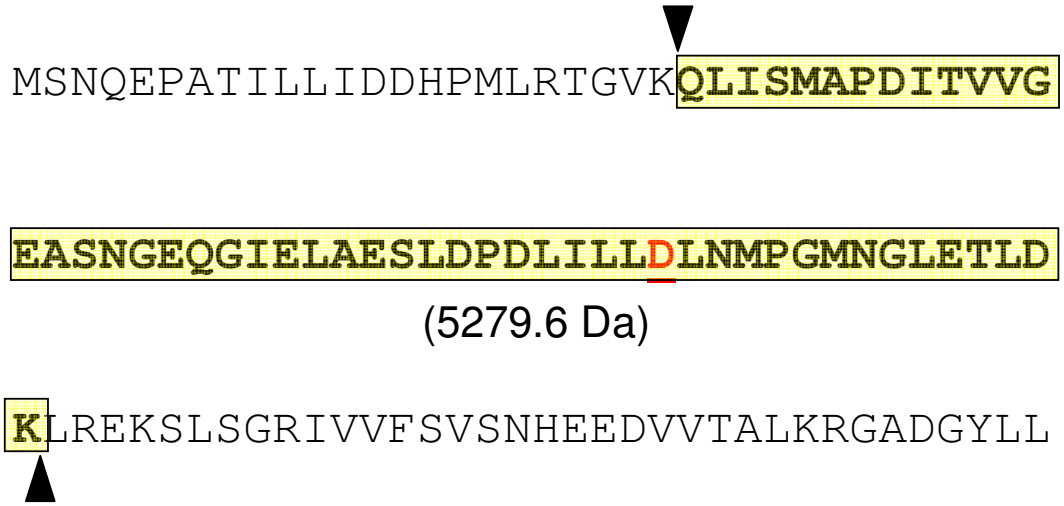

Figure 6.4 *In silico* trypsin digestion of NarL and identification of phospho-peptide

The NarL polypeptide, when treated with trypsin, would yield a number of peptides, including a 5.3 kDa peptide (bold boxed sequence) that contains the protein phosphorylation site, Asp 59 (red underlined). This *in silico* peptide digest was generated using PeptideCutter, available at http://www.expasy.ch/tools/peptidecutter

procedure was identical to the protocol used for the DNA-sampling experiment described in chapter 5, with the addition of DNAse to the lysis buffer.

All attempts at this experimental method yielded high quantities of NarL-FLAG protein, which was separated by SDS-PAGE and visualised using SilverQuest® silver staining (Invitrogen)(figure 6.5). Proteins were then destained, digested with trypsin and eluted from the gel. Eluted peptides were analysed on a Thermo-Finnigan FT-ICR mass spectrometer using a NanoMate chip-based electrospray system operated by the University of Birmingham Functional Genomics and Proteomics Unit. On all occasions, the protein purified was identified as NarL by its peptide sequence (data not shown). However, despite several attempts, it was not possible to identify the peptide containing the phosphorylation site, most likely due to its relatively large size (5.2 kDa).

This experiment may be a good way of determining whether FNR indirectly regulates NarL dependent promoter activity by modulating NarL-phosphate levels. However, modifications will need to be made in order to maximise the chance of identifying the correct peptide. In the future, site directed mutagenesis could be used to introduce a recombinant trypsination site proximal to Asp59, to facilitate the identification of the phospho-peptide.

The physiological roles of the gene products of yeaR-yoaG, ogt and hcp-hcr

Nitrate regulation of DNA repair

The physiological role of Ogt has been widely and thoroughly studied. It is accepted that the *ogt* gene encodes an O^6-alkylguanine DNA-alkyltransferase that removes methyl groups from alkylated DNA (Margison *et al.*, 1985; Potter *et al.*, 1987). However, this study and the work of Meng Xu (PhD thesis) provide the first evidence of nitrate regulation of *ogt* expression, and hence of DNA repair (Squire *et al.*, 2009). The rationale for this unprecedented regulation comes from the fact that RNS, in particular NO, are known to cause an indirect

Figure 6.5

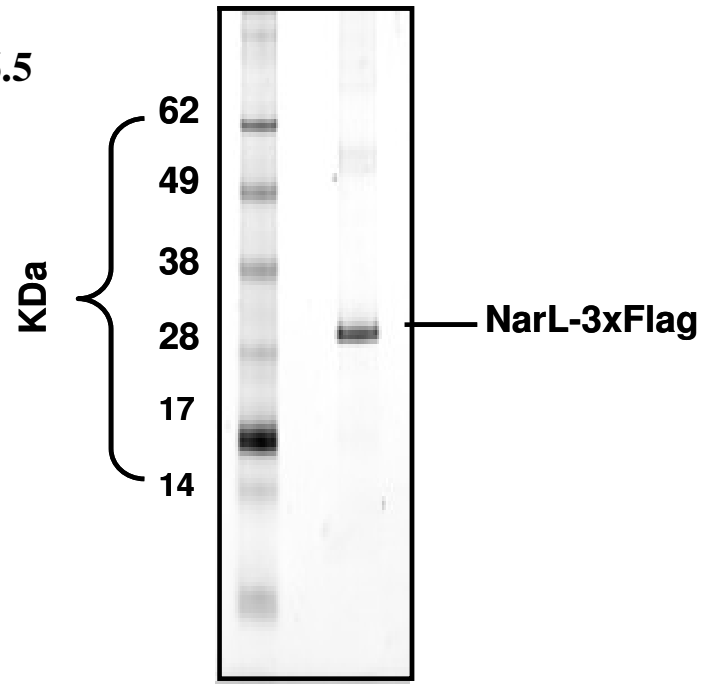

Fig 6.5 Affinity-directed isolation of NarL-3xFLAG

Strain JCB387Δ*narL-3x*FLAG was grown anaerobically to an OD_{650} of 0.5 in the presence of 2.5 mM nitrite. Cells were harvested, lysed by sonication and cell extract was obtained. Promoter NarL-FLAG protein was isolated by incubation with anti-FLAG conjugated magnetic beads, washed and eluted. Proteins were separated by SDS-PAGE on a 4-12% gradient gel, calibrated for size with SeeBlue® prestained protein markers.

A single high intensity band, with the correct molecular weight for NarL-3xFLAG, was excised, de-stained, and digested with porcine trypsin. Peptides were then eluted and analysed on a Thermo-Finnigan FT-ICR mass spectrometer using a NanoMate chip-based electrospray system, run by the Functional Genomics Suite at the University of Birmingham.

increase in DNA mutation rates (Weiss, 2006). In the presence of NO, lysine, other amino acids and other chemicals, such as methylamine, can become potent DNA methylating agents (Kunisaki and Hayashi, 1979; Swann and Magee, 1968; Taverna and Sedgwick, 1996). The regulation of *ogt* in response to nitrate, via NarL alone, indicates that Ogt is synthesised as part of a prophylactic response to endogenous RNS that are generated during anaerobic nitrate and nitrite metabolism (Squire *et al.*, 2009; Weiss, 2006). Surprisingly, S-adenosyl methionine (SAM) was shown not to be the major DNA-methylating mutagen in these nitrosative stress conditions (Taverna and Sedgwick, 1996).

Repair of iron-sulphur centres damaged by nitric oxide

The product of the *E. coli ytfE* gene has been shown to have an important role in resistance to nitric oxide stress (Justino *et al.*, 2005b; Justino *et al.*, 2007). Like *yeaR-yoaG*, *ytfE* has been shown to be repressed by NsrR (Filenko *et al.*, 2007). Bioinformatic analysis of the promoter region has also identified a putative FNR binding site that overlaps the RNA polymerase binding site. The expression patterns of *ytfE* and *yeaR-yoaG* were also very similar in two independent transcriptomic studies that measured the expression of genes in response to nitrate, nitrite or nitric oxide (Constantinidou *et al.*, 2006; Justino *et al.*, 2005b). The fact that both of these genes are regulated in a very similar fashion by a very similar set of transcription factors suggests that the products of both genes may be involved in similar or even the same pathways.

Much of the physiological data published on the growth phenotypes of a *ytfE* null mutant and its ability to grow under different anaerobic conditions is now being reviewed due to the identification of a chromosomal deletion of approximately 100 genes in the *ytfE* mutant strain (Claire Vine. unpublished data). However, the gene homologue of *ytfE* in *Neisseria gonorrhoeae, dnrN,* has been shown to be involved in the repair of the transcription factor, FNR, after damage by nitric oxide (Overton *et al.*, 2008). Published evidence shows that

purified YtfE can repair the metabolic enzymes, Aconitase B and Fumarase A, after nitric oxide damage (Justino *et al.*, 2006). However, whether this repair is due to the reduction or removal of NO from the iron-sulphur centres of these proteins, or the re-insertion of an entirely new iron-sulphur centre, remains to be proven. Whether YtfE is able to repair iron-sulphur centre containing transcription factors, such as FNR, in *E. coli* is currently being investigated. A previous investigation into the repair of the iron-sulphur containing *E. coli* ferredoxin, following nitrosative stress, revealed that the restoration of ferredoxin function was not due to *de novo* synthesis but to the repair of the damaged dinitrosyl iron cluster. Furthermore, this repair was shown to be dependent upon the availability of cellular L-cysteine (Rogers and Ding, 2001).

When *E. coli* encounters nitric oxide, damage to the cell is inevitable due to the highly reactive nature of nitric oxide. Amongst the many biological components to which nitric oxide can bind are iron-sulphur centres that are essential components of many enzymes, involved in all aspects of metabolism. When nitric oxide binds to iron-sulphur centres, a nitrosyl adduct is formed (fig 6.6). How these damaged iron-sulphur centres are repaired is unknown, but it could involve either the reduction of the nitrosyl adduct *in situ* or the excision or transfer of the nitrosyl group to another recipient. If the nitroso-adduct was reduced *in situ*, while still bonded to the iron atom, it is possible that several reduction steps, catalysed by several enzymes might be required to remove the NO group entirely. One scheme proposed is that the nitrosyl adduct would be reduced to a metal-bound hydroxamate adduct by an enzyme-catalysed 2 electron reduction, before being reduced to hydroxylamine by a further reduction, possibly involving a second enzyme (fig.6.7). The hydroxylamine produced from this reduction could then be reduced by Hcp or another unknown enzyme. Alternatively, a transferase enzyme could catalyze the removal of the NO adduct entirely to another protein, most likely a thiol-containing enzyme, in a process known as transnitrosation (Arnelle and Stamler, 1995). Under the right physiological conditions, transnitrosation of the metal bound nitroso-adduct is

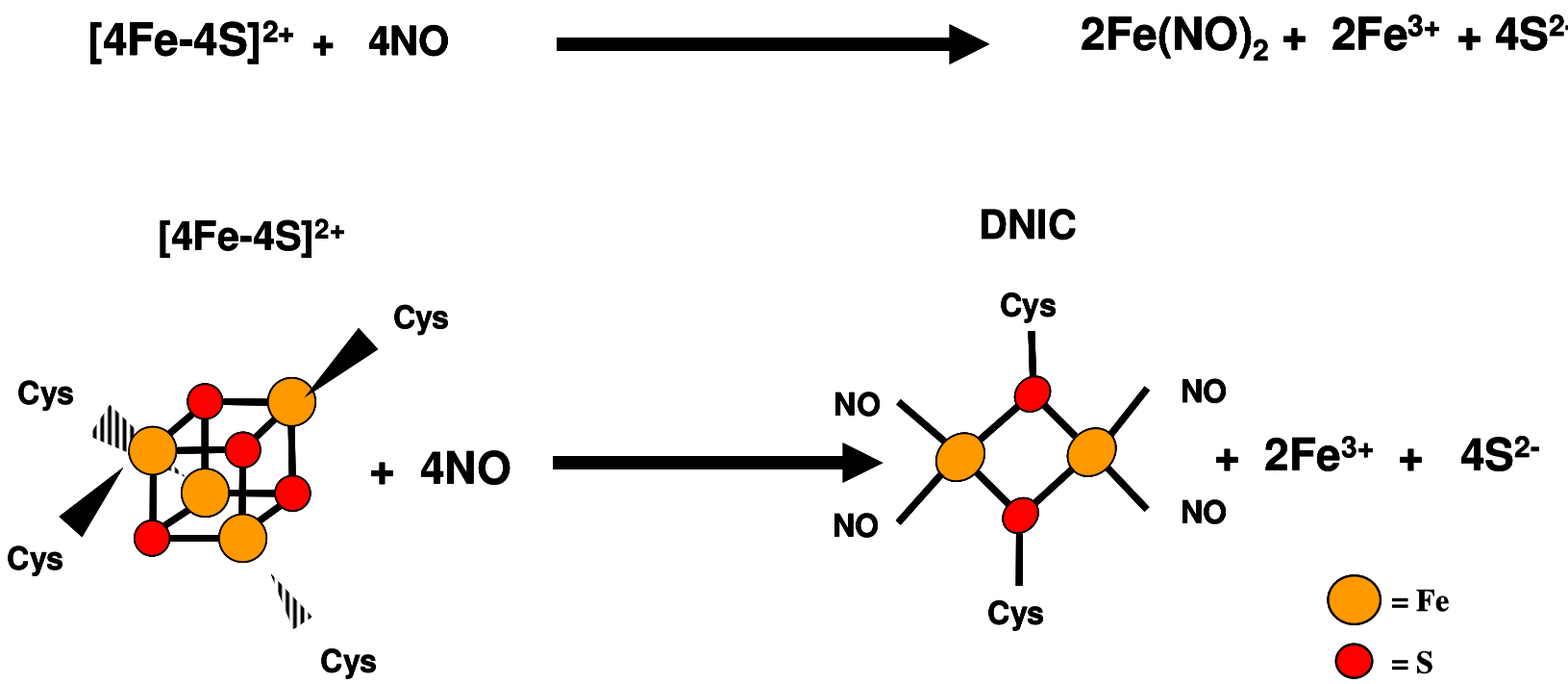

Figure 6.6 The reaction of NO with [4Fe-4S] iron-sulphur clusters

The $[4Fe\text{-}4s]^{2+}$ iron sulphur centre of FNR is nitrosylated upon exposure to nitric oxide, resulting in the formation of a dinitrosyl-iron complex (DNIC), and the liberation of two ferric iron atoms and four sulphide ions. When the iron-sulphur centre of FNR in nitrosylated to form a DNIC, the protein is unable to dimerise and is inactive.

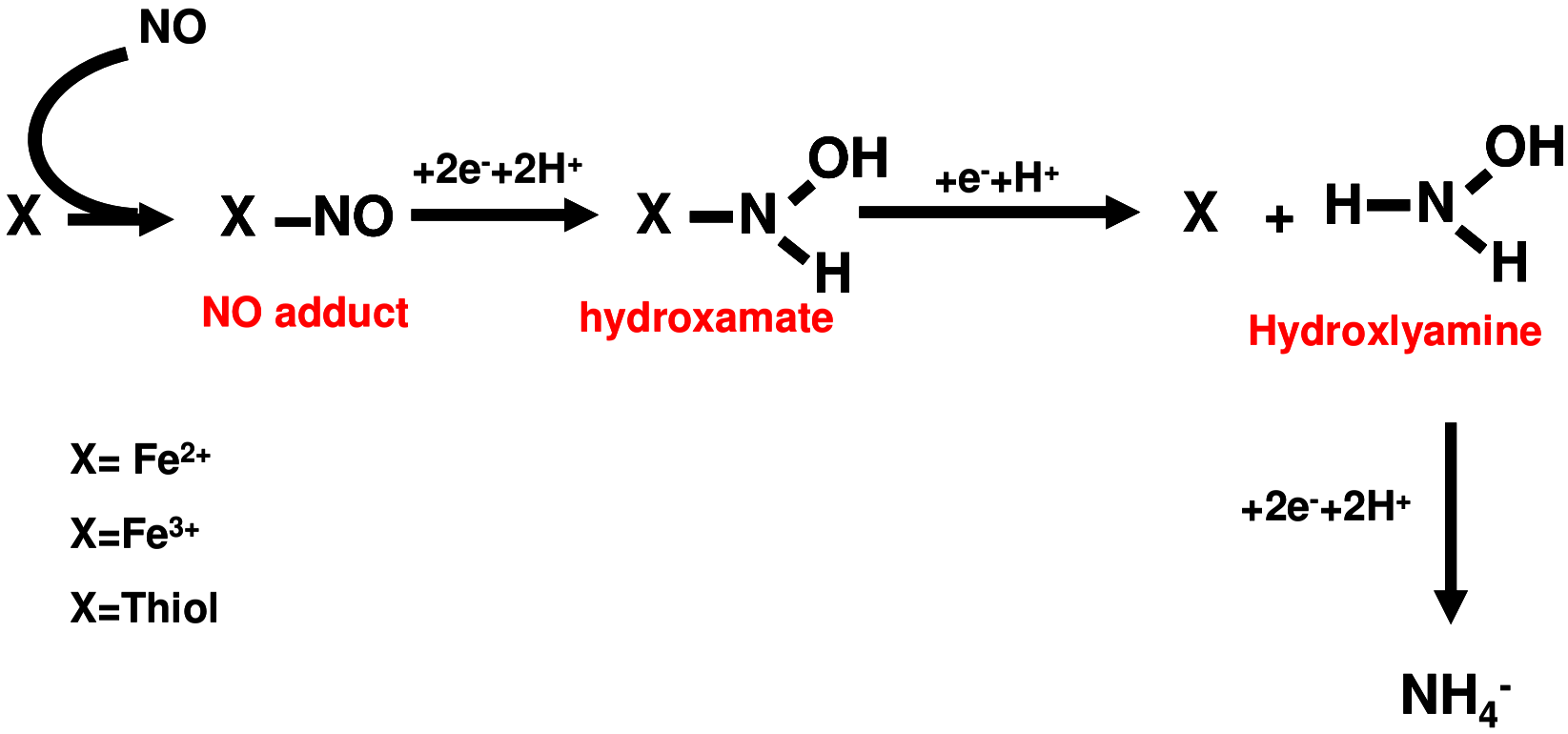

Figure 6.7 A theoretical detoxification pathway for damaged, nitrosated cellular components

In this theoretical scheme a nitroso-adduct is reduced to a hydroxamate by a 2 electron reduction then to free hydroxylamine by a further 1 electron reduction. Hydroxylamine is then reduced to ammonia and water by a final 2 electron reduction. The first two reduction steps could be catalysed by any of the genes of unknown function implicated in nitrosative stress. The final reduction of hydroxylamine to ammonia could be catalysed by HCP or NirB. It is likely that for each different damaged cell component e.g. thiol or metal centre, that a different repair pathway would exist.

possible, but this would still leave the cell with the problem of having to remove the nitrosyl adduct from a different protein. One possible, but metabolically unfavourable, mechanism of NO removal would be for the cell to synthesise a protein that would be used as an 'NO-sponge', acting as a target for nitrosation by NO or transnitrosation before being degraded entirely. Any protein synthesized during conditions of nitrosative stress could fulfil this role. However, a highly expressed protein, especially one containing an iron-sulphur, centre such as Hcp, would be an ideal candidate.

Structural domain similarity of YeaR and TehB

Bio-informatic analysis of the YeaR protein sequence, using domain specific BLAST searching, PSI-BLAST, revealed the presence of a DUF1971 superfamily domain in the YeaR amino acid sequence. This domain is most often found in the tellurite resistance protein, TehB, from several organisms. The highest similarity score for a protein of known structure was TehB of *Vibrio fischeri* ES114 with a hit score of 85.5 and an e value of $2e^{-16}$. This result is considered to be significant for elucidating the function of YeaR for several reasons. As has been shown in previous sections, nitric oxide toxicity often involves the binding of NO to metal groups, or the conversion of NO to secondary RNS in the presence of metals. How TehB functions to remove tellurium from the cytoplasm is not known, but the protein has been shown to contain a conserved motif known as the S-adenosylmethionine (SAM)-dependent non-nucleic acid methyltransferase motif, and site-directed mutagenesis of conserved residues cause loss of tellurite resistance. TehB over-expressed from a plasmid has been shown to facilitate the loss of TeO_3^{2-} from an assay mixture, but the reaction end-product is not known (Liu *et al*., 2000). Whether or not YeaR possesses this same SAM-binding motif has not been determined at present. Tellurium is found in the same group of the periodic table as the essential element, selenium, which is incorporated into the modified amino acid, seleno-cysteine. The similar chemical properties of these two elements might suggest that YeaR could

be involved in the processing of selenium in response to NO stress. How cellular selenium levels are affected by NO, or the reaction products that occur, are not known but would be of interest to this study. In some bacterial species, YeaR is present as an amino-terminal extension of TehB and thus is often referred to as TehB or TehB^ (Lin *et al.*, 2007; Rodionov *et al.*, 2005). In the Vibrionales and some Enterobacteria, *tehB* or *tehB^* are encoded on the chromosome immediately downstream of the nitric oxide reductase encoding genes, *norVW*, and are presumed to be co-ordinately expressed in response to nitric oxide (Rodionov *et al.*, 2005). Finally, the cryptic tellurite resistance protein of *E. coli*, TehB, which shows only limited similarity to YeaR, was shown to be upregulated in the presence of NO and also nitrite (Constantinidou *et al.*, 2006; Justino *et al.*, 2005b). A putative NsrR binding site was identified in the promoter of TehB, situated in a position that overlaps the transcription start site (Bodenmiller and Spiro, 2006). Why tellurite resistance or related cell processes might be regulated by nitric oxide stress is not understood, but offers new possibilities for the function of YeaR and YoaG.

Nitrogen isotope, N^{15}, tracer experiments to identify nitric oxide detoxification pathways

In a recent investigation into the properties and function of the nitrate and nitrite transporter in *E. coli*, NarU, the heavy isotope N^{15} was used to track the accumulation of nitrite and ammonia inside and outside the cell during growth in the presence of nitrate (Jia *et al.*, 2009). Using highly accurate mass spectrometry, the heavier, non-radioactive, isotope can be identified even in chemically different species, such as nitrate and nitrite, by the difference in molecular mass. This procedure could be used to identify the pathway for nitric oxide detoxification, especially from iron-NO adducts, as have been discussed earlier. The isotope, N^{15}, is not available in the form of nitric oxide gas but N^{15} nitric oxide could be generated from N^{15} nitrite (Sigma Aldrich) in an acidic environment, as has been shown previously (Kim *et al.*, 2003). The accumulation of different pathway products, such as hydroxylamine and

hydroxamates, could then be investigated in mutants lacking one or more of the genes implicated in NO detoxification. This technique could lead to the identification of the physiological function of one or more genes of unknown function in the resistance of *E. coli* to reactive nitrogen species.

The hypothetical function of YeaR-YoaG and its role in nitrosative stress

This study has presented clear evidence that the operon of unknown function *yeaR-yoaG* and the gene *ogt* are regulated in response to nitrate, via NarL, by an FNR independent mechanism. As has been discussed, nitrate and nitrite reduction, which are utilised by the bacterium to survive in anaerobic conditions, can lead to the generation of highly cytotoxic nitric oxide (Corker and Poole, 2003). The generation of nitric oxide from nitrate and nitrite metabolism would undoubtedly disrupt the cell and would eventually de-activate the global response regulator, FNR, by binding to the essential iron-sulphur centre and forming a stable dinitrosyl adduct (Cruz-Ramos *et al.*, 2002). Under these conditions, it would be expected that a number of SOS proteins would be synthesised in order to deal with the nitric oxide present and the damage it has caused (Constantinidou *et al.*, 2006; Justino *et al.*, 2005b). A mechanism of direct regulation by NO, via FNR, has already been proven at the *hmpA* promoter, which encodes the flavohaemaglobin, Hmp, a nitric oxide detoxification system. Active FNR binds to the *hmp* promoter and represses transcription (Cruz-Ramos *et al.*, 2002). When nitric oxide is produced, it deactivates FNR, leading to Hmp synthesis and nitric oxide detoxification. In contrast to p*hmp*, the regulation of *yeaR* is only indirectly effected by FNR, suggesting that YeaR-YoaG might be synthesised as a prophylactic response to the nitric oxide precursor, nitrate.

The role of NsrR at the *yeaR* promoter is vital to understanding whether the gene is expressed in response to, or in preparation for, nitrosative stress. A single binding site for NsrR exists in the promoter region of *yeaR,* and a 4-fold effect of NsrR repression on *yeaR*

transcription was reported (Filenko *et al.*, 2007; Lin *et al.*, 2007). However, this study has demonstrated that the apparent role of NsrR *in vivo* is relatively weak. The experiments suggested earlier in this discussion, to ascertain whether NsrR binds to the *yeaR* promoter, would be vital to identifying the physiological role of the YeaR-YoaG proteins.

In general, when studying bacterial physiology, understanding the regulation of a gene is key to understanding the function of the encoded proteins. The conditions when proteins are maximally expressed tend to be the conditions where the encoded protein is most active and most needed. Furthermore, groups of proteins that are required in response to different environmental conditions are often co-regulated and under the control of similar transcription factors (Browning and Busby, 2004; Constantinidou *et al.*, 2006). These assumptions suggest that *yeaR-yoaG*, which is expressed in response to nitrate, and is co-regulated with genes known to be involved in nitrosative stress management, forms part of a response to nitrosative stress.

Despite the implication that *yeaR-yoaG* forms part of an SOS response to deal with nitric oxide, no clear growth inhibition phenotype of a *yeaR-yoaG* null strain by nitric oxide has been observed. This suggests that the function of YeaR-YoaG is not vital to the ability of *E. coli* to grow in these conditions. This apparent redundancy appears to be common throughout nitrosative stress responses, as the *E. coli* chromosome encodes three proteins with known nitric oxide reductase capabilities (HmpA, NorVW and NrfA). It is not likely that these systems are functionally redundant, but that they are synthesised under subtly different conditions, for example, when exogenous nitric oxide is encountered as opposed to endogenous NO. However, the presence of several 'back-up' systems, even if differently expressed, makes the identification of clean growth phenotypes in response to nitric oxide difficult. In order to understand the contribution that each of these systems makes to nitric oxide reduction/ detoxification it would be necessary to construct mutants that are deficient in all combinations of these known systems, as well as genes implicated in nitrosative stress management. A

systematic investigation into the contribution of, HmpA, NorVW, NrfA, NirB, Hcp and YeaR-YoaG to the rate of nitric oxide reduction by *E coli* grown in different anaerobic conditions is being undertaken by Claire Vine of the University of Birmingham, which will aid in the discovery of the function of YeaR-YoaG.

The existence genes encoding of several other systems for nitric oxide reduction on the *E. coli* chromosome and the lack of growth phenotype during nitrosative stress lead to the proposal that YeaR-YoaG is not a further nitric oxide reductase. One other possible indication of the function of YeaR-YoaG comes from the regulation of *hmpA*. The promoter region of *hmpA* contains two operators for the MetR transcriptional regulator, a global regulator that controls genes involved in methionine biosynthesis. The DNA-binding affinity of MetR is controlled by the availability of homocysteine, which binds to the MetR regulator and modifies its activity (Urbanowski, 1989). In the presence of homocysteine, MetR binds to both of the operators in the *hmp* promoter region and represses transcription (Membrillo-Hernandez *et al.*, 1998). When homocysteine levels are depleted, only the MetR site proximal to the -35 of p*hmp* is occupied and transcription from p*hmp* is activated. The cellular levels of the MetR cofactor, homocysteine, and the synthesis of methionine are dramatically effected by the addition of GSNO, a potent NO releasing compound, which nitrosates homocysteine to form S-nitrosohomocysteine (Flatley *et al.*, 2005).

The dependence of Hmp expression on homocysteine availability raises a possible route of investigation for the function of YeaR-YoaG. Homocysteine is an integral component of the cyclical methionine biosynthetic pathway (fig 6.8). This pathway also involves the formation of S-adenosylmethionine (SAM), a potent methylating agent. As noted previously, it has been demonstrated that YeaR shares significant sequence and domain similarity with the tellurite resistance protein, TehB, whch is also appears to be regulated by nitrosative stress (Constantinidou *et al.*, 2006; Justino *et al.*, 2005b). The function of TehB and related proteins in many bacteria is thought to be dependent upon the methylation of tellurite by TehB and the

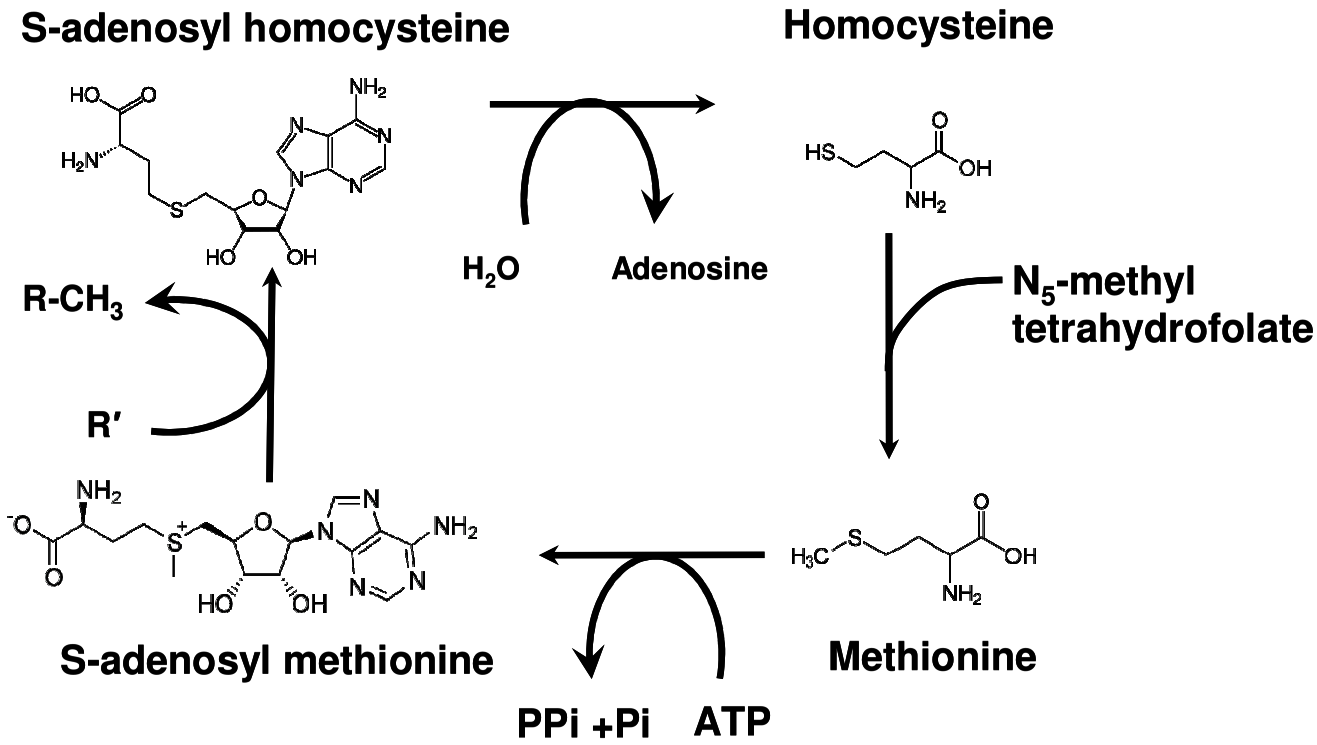

Figure 6.8 The methionine biosynthetic pathway and 1-carbon metabolism in *E. coli*

Methionine is synthesised by *E. coli* in a cyclical pathway from homocysteine. A single methyl group is donated by methyl tetrahydrofolate to the thiol group of homocysteine to form methionine. Methionine is broken down by the addition of an adenosyl group from ATP, yielding S-adenosyl methionine (SAM), a potent methylating agent. The loss of the methyl group from SAM leads to the formation of S-adenosyl homocysteine (SAH), which can be further hydrolysed to regenerate homocysteine.

protein bound co-factor SAM. It could be feasible that YeaR is also able to coordinate SAM, or another structurally related product, that accumulates during growth in nitrosative stress conditions due to the disruption of the methionine biosynthetic pathway. An investigation into the cellular levels of homocysteine, SAM and methionine, as well as other metabolites such as N_5-methyl tetrahydrofolate, during nitrosative stress might aid in the discovery of YeaR-YoaG function. The use of chemostat cultures and small molecule metabolomics might offer an insight into the downstream effects of nitrosative stress on bacterial metabolism.

The structure of YeaR has recently been resolved by X-ray crystallography by the Northeast Structural Genomics Consortium (http://www.nesg.org/). The protein exists as a tetramer with a propeller-like structure, with each YeaR protein chain incorporating a zinc ion as a chemical ligand (fig 6.9). This information might assist in the determination of YeaR function in the cell.

Final conclusions

The conclusions of this study are that the regulatory mechanisms of *yeaR* and *ogt* offer new insights into the possible functions of the proteins encoded by these genes in nitrosative stress tolerance. The ability of *E. coli* to resist the damaging effects of nitric oxide and other reactive nitrogen species is vital to its ability to thrive in the environment and in the gut of mammals. The activation of these proteins by NarL, independent of FNR, reveals a new level of complexity in how *E. coli* is able to respond to the changing environment it encounters during infection, and might offer new insight into the pathogenicity of *E. coli* strains. Although several possible explanations for the increased nitrite-dependent *yeaR* promoter activity in the absence of functional FNR have been inverstigated, and future experiments suggested, it most likely that this effect is due to a lack of nitrate and nitrite metabolism in an *fnr* null mutant, leading to an abundance of inducer molecule (nitrite) and thefore increase NarL activity.

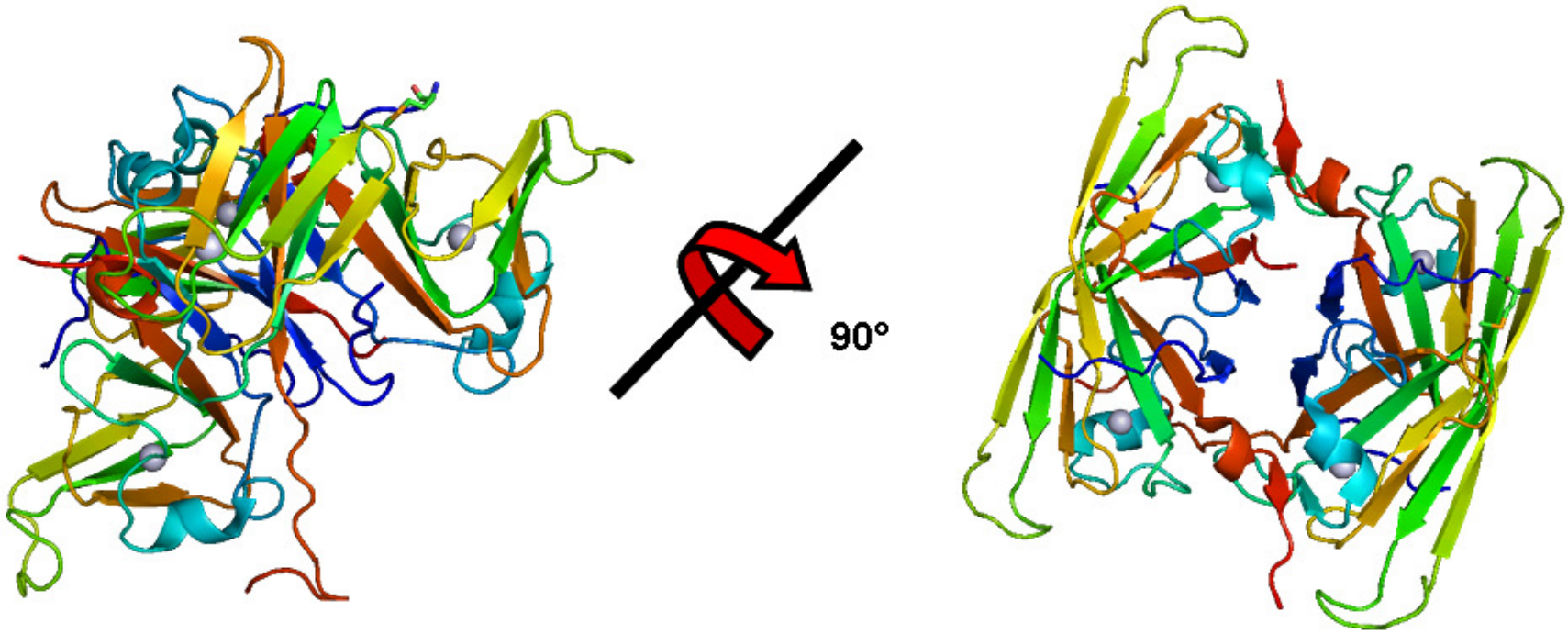

Figure 6.9 The 3D structure of YeaR

The 3D structure of YeaR was resolved by X-ray crystallography to a resolution of 2.3 Å. The YeaR protein exists as a tetramer, with each protein chain co-ordinating one zinc atom as a chemical ligand (grey spheres). This structure was obtained from the RSCB protein data bank (accession number 3bb6) and was modelled using PyMol software.

However, this would indicate that p*yeaR* and p*ogt* are subject to unusal transcriptional control by NarL, which would normally be de-phosphorylated by NarX in response to nitrite.

Despite the lack of a growth phenotype during nitrosative stress, it is certain that YeaR and other proteins expressed in these conditions, such as Hcp, have a very important role in dealing with the damage and disruption caused to the cell by nitric oxide, if not detoxifying the molecule itself. It is proposed that the function of YeaR is to deal with a downstream effect of nitric oxide, rather than to reduce nitric oxide directly. This could involve the repair of iron-sulphur centres, the removal of NO from thiol groups, or the regeneration of a nitrosylated metabolite such as homocysteine. This study also prompts many other lines of investigation into how *E. coli* regulates transcription during stress conditions, and how these transcriptional networks affect the lifestyle of *E. coli*.

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1. Filenko, N., Spiro, S., Browning, D.F., Squire, D., Overton, T.W., Cole, J., and Constantinidou, C. (2007) The NsrR regulon of *Escherichia coli* K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. *J Bacteriol* **189**: 4410-4417

2. Squire, D.J., Xu, M., Cole, J.A., Busby, S.J., and Browning, D.F. (2009) Competition between NarL-dependent activation and Fis-dependent repression controls expression from the *Escherichia coli yeaR* and *ogt* promoters. *Biochem J* **420**: 249-257.