Characterization of the *Escherichia coli* K-12 *ydhYVWXUT* operon: regulation by FNR, NarL and NarP

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In *Escherichia coli* K-12 the expression of many genes is controlled by the oxygen-responsive transcription factor FNR and the nitrate- and nitrite-responsive two-component systems NarXL and NarPQ. Here, the *ydhY* gene is shown to be the first gene of a six-gene operon (*ydhYVWXUT*) that encodes proteins predicted to be components of an oxidoreductase. Mapping the *ydhY*–*T* transcript start and site-directed mutagenesis confirmed that the *ydhY*–*T* genes are transcribed from an FNR-dependent class II promoter and showed that the FNR site is centred at –42.5. In the presence of nitrate or nitrite, NarXL and NarPQ repressed *ydhY*–*T* expression. Analysis of the DNA sequence of the *ydhY* promoter region (*PydhY*) revealed the presence of four heptameric sequences similar to NarL/P binding sites centred at –42, –16, +6 and +15. The latter heptamers are arranged as a 7-2-7 inverted repeat, which is required for recognition by NarP. Accordingly, NarP protected the 7-2-7 region in DNase I footprints, and mutation of either heptamer +6 or heptamer +15 impaired nitrite-mediated repression, whereas mutation of heptamer –42 and heptamer –16 did not affect the response to nitrite. The NarL protein also protected the 7-2-7 region, but in contrast to NarP, the NarL footprint extended further upstream to encompass the –16 heptamer. The extended NarL footprint was consistent with the presence of multiple NarL–*PydhY* complexes in gel retardation assays. Mutation of heptamer –42, which is located within the FNR binding site, or heptamer +6 (but not heptamers –16 or +15) impaired nitrate-mediated repression. Thus, although the region of the *ydhY*–*T* promoter containing the –16 and +15 heptamers was recognized by NarL *in vitro*, mutation of these heptamers did not affect NarL-mediated repression *in vivo*.

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INTRODUCTION

Escherichia coli K-12 is a facultative anaerobe that can utilize a range of alternative electron acceptors to maintain respiration in the absence of the preferred electron acceptor, oxygen. However, in the absence of oxygen, nitrate or nitrite is utilized in preference to other alternative electron acceptors such as fumarate, DMSO or trimethylamine *N*-oxide (Guest *et al.*, 1996; Unden *et al.*, 2002). This metabolic hierarchy reflects the amount of energy that can be conserved by each process, and is maintained by oxygen-, nitrate- and nitrite-responsive transcription factors. The *E. coli* FNR protein is an oxygen-responsive global transcription factor (Kiley & Beinert, 2003). Under anaerobic conditions FNR contains a

[4Fe–4S] cluster that promotes dimerization of the protein and enhances site-specific DNA binding (Jervis & Green, 2007; Lazazzera *et al.*, 1996). In the presence of oxygen, disassembly of the FNR iron–sulphur clusters is accompanied by the formation of FNR monomers and inhibition of DNA binding, thereby switching off FNR-activated genes (Kiley & Beinert, 2003; Lazazzera *et al.*, 1996; Sutton *et al.*, 2004). The DNA sequence recognized by FNR consists of an inverted repeat with a consensus sequence of TTGATnnnnATCAA (Eiglmeier *et al.*, 1989). Once bound to DNA, FNR dimers activate transcription by recruiting RNA polymerase, or alternatively repress transcription by inhibiting the formation of productive promoter–RNA polymerase interactions (reviewed by Browning *et al.*, 2003). There are two basic types of FNR-dependent promoter. Class I promoters possess FNR sites located close to –61.5, –71.5, –82.5 or –92.5 (Wing *et al.*, 1995).

Abbreviations: MBP, maltose-binding protein; RACE, random amplification of cDNA ends.

This architecture permits the formation of only one protein–protein contact, between FNR and the C-terminal domain of the RNA polymerase α -subunit (Browning *et al.*, 2003). However, the more common architecture for FNR-activated promoters is that adopted by class II promoters, which have an FNR site centred at or close to 41.5 bp upstream of the transcript start site. This arrangement allows the formation of multiple protein–protein contacts between RNA polymerase and FNR (Browning *et al.*, 2003).

As mentioned above, in the absence of oxygen, nitrate is the preferred electron acceptor (Guest *et al.*, 1996). In *E. coli* the presence of nitrate is sensed by paralogous two-component systems NarXL and NarPQ (Stewart, 1994). The membrane-bound sensors NarX and NarP perceive the presence of nitrate and nitrite in the environment (Lee *et al.*, 1999; Rabin & Stewart, 1992, 1993; Williams & Stewart, 1997). These sensors then act to control the phosphorylation state of the regulators NarL and NarP (Darwin & Stewart, 1996; Yamamoto *et al.*, 2005), and there is a complex exchange of information between the sensors and the regulators to allow fine control of target-gene expression in response to nitrate and nitrite. The phosphorylated regulators bind specific DNA sequences related to the consensus TACYMT (where Y=C or T, and M=A or C) to positively or negatively regulate gene expression (Darwin *et al.*, 1997). In addition, there is differential recognition of NarL/P heptamer patterning, such that NarL recognizes all TACYMT heptamers, whereas NarP only binds these sequences when they are arranged as an inverted repeat with a 2 bp spacing, the so-called 7-2-7 arrangement (Darwin *et al.*, 1997). Thus, the Nar two-component systems work in conjunction with FNR to maintain a central metabolic hierarchy in *E. coli*. For example, in the absence of oxygen, nitrate promotes the NarL-mediated activation of the FNR-dependent nitrate reductase operon (*narGHJI*) and NarL-mediated repression of the FNR-dependent fumarate reductase operon (*frdABCD*) (Darwin & Stewart, 1996).

Upstream of the *E. coli ydhY* gene is a consensus FNR site. Transcript-profiling experiments have shown that the abundance of the *ydhY* and downstream transcripts is enhanced under anaerobic conditions in the presence of FNR (Constantinidou *et al.*, 2006; Kang *et al.*, 2005), and that the transcripts are less abundant in the presence of nitrate (Constantinidou *et al.*, 2006). *In vivo* transcription studies using a *ydhY*–*lacZ* fusion have shown that *ydhY* expression is FNR-dependent, and *in vitro* transcription reactions in the presence of FNR yield a product with a size consistent with the presence of a class II FNR-dependent promoter upstream of *ydhY* (Kang *et al.*, 2005).

The amino acid sequences of the proteins encoded by the *ydhY*–*T* operon suggest that they are components of an oxidoreductase. Thus, YdhX (222 aa) is a putative electron transfer protein with a Tat signal sequence (MSFTRR-KFVLGMGTVIFFTGASLLA) (Birks *et al.*, 2005), four

[4Fe–4S] clusters and 50 % identity over 213 aa to the nitrite reductase protein NrfC from *E. coli*. The YdhV protein (700 aa) possesses DXXGL motifs that are associated with archaeal tungsten-containing oxidoreductases and is 28 % identical (41 % similar over 687 aa) to the aldehyde ferredoxin oxidoreductase of *Pyrococcus furiosus* (Kletzin *et al.*, 1995). This protein lacks a Tat signal sequence and thus should be located in the cytoplasm, unless it is exported as a complex with YdhX. YdhU (261 aa) is a predicted cytochrome *b*-containing integral membrane protein that is 43 % identical to the thiosulphate reductase cytochrome *b* subunit of *Salmonella typhimurium* (Heinzinger *et al.*, 1995). For Tat substrates such as YdhX, the assembly of the catalytic complex requires accessory proteins known as redox enzyme maturation proteins (REMPs), which are often co-transcribed with the genes encoding the oxidoreductase subunits (Turner *et al.*, 2004). It is possible that the YdhW and YdhT proteins are REMPs. The YdhY protein (208 aa) is predicted to be ferredoxin-like in possessing four iron–sulphur clusters. Thus, it is possible that YdhY is involved in electron transfer reactions with YdhV.

In this study, it is shown that the *ydhY* gene is the first gene of a six-gene operon (*ydhYVWXUT*). The mechanism of regulation of *ydhY*–*T* expression by FNR, in response to oxygen availability, and by the NarXL and NarPQ systems, in response to nitrate and nitrite availability, revealed that NarL represses *ydhY*–*T* expression by binding to sites in the promoter region that overlap those occupied by FNR and RNA polymerase. Furthermore, it is shown that NarP recognizes a 7-2-7 site close to the transcript start to repress *ydhY*–*T* expression in the presence of nitrate and nitrite, providing new evidence in support of earlier transcript-profiling experiments that suggest that nitrate-activated NarP can act as a repressor (Constantinidou *et al.*, 2006).

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this work are listed in Table 1. Cultures of *E. coli* were grown in L broth (Lennox, 1955) or Evans defined minimal medium (Evans *et al.*, 1970) supplemented with appropriate antibiotics (ampicillin, 100 $\mu\text{g ml}^{-1}$; chloramphenicol, 20 $\mu\text{g ml}^{-1}$; tetracycline, 25 $\mu\text{g ml}^{-1}$). For β -galactosidase assays, L broth containing appropriate antibiotics was inoculated (1:50) from overnight cultures and incubated under aerobic (250 ml conical flasks containing 5 ml medium shaken at 250 r.p.m.) or anaerobic (sealed bottles filled to the neck) conditions at 37 °C until OD₆₀₀ 0.4–0.6 was reached. β -Galactosidase activities were then measured (Miller, 1972).

Nucleic acid methods. DNA was isolated and manipulated by conventional methods (Sambrook & Russell, 2001). The *ydhY* promoter region (–189 to +176 relative to the transcription start) was amplified from the chromosome of *E. coli* MC1000 by PCR using appropriate oligonucleotides designed to contain *EcoRI*/*Bam*HI restriction sites. The authenticity of the amplified fragment was verified by DNA sequencing after ligation into *EcoRI*/*Bam*HI-digested pRS415 to create pGS1739 (Simons *et al.*, 1987). The inserts were then transferred to λ RZ5 and the resulting (promoter–*lacZ*) fusions were

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant genotype or properties	Reference or source
<i>E. coli</i> strains		
DJ901	GC4468 <i>soxRS</i>	Greenberg <i>et al.</i> (1990)
GC4468	$\Delta(\arg F-lac)U169$ <i>rpsL sup</i>	Greenberg <i>et al.</i> (1990)
GS047	MC4100 <i>oxyR-Kan</i>	Zheng <i>et al.</i> (1999)
JRG1728	MC1000 $\Delta(\text{tyrR-fnr-rac-trg})17$ <i>zdd-230-Tn9</i>	Spiro & Guest (1987)
JRG5146	MC1000/ λ RZ5(<i>PydhY-lacZ</i>) native promoter derived from pGS1739	This study
JRG5147	JRG1728/ λ RZ5(<i>PydhY-lacZ</i>) native promoter derived from pGS1739	This study
JRG5199	W3110 <i>ydHVVWXUT</i>	This study
JRG5611	VJS691/ λ RZ5(<i>PydhY-lacZ</i>)	This study
JRG5612	VJS1357/ λ RZ5(<i>PydhY-lacZ</i>)	This study
JRG5613	VJS691 <i>narQ251-Tn10d narP253::Tn10d</i> (Cm ^R) / λ RZ5(<i>PydhY-lacZ</i>)	This study
JRG5614	VJS1357 <i>narQ251-Tn10d narP253::Tn10d</i> (Cm ^R) / λ RZ5(<i>PydhY-lacZ</i>)	This study
JRG5647	MC1000/ λ RZ5(<i>PydhY-lacZ</i>) derived from pGS2010	This study
JRG5648	MC1000/ λ RZ5(<i>PydhY-lacZ</i>) derived from pGS2011	This study
JRG5649	MC1000/ λ RZ5(<i>PydhY-lacZ</i>) derived from pGS2012	This study
JRG5650	MC1000/ λ RZ5(<i>PydhY-lacZ</i>) derived from pGS2013	This study
M182	$\Delta(\text{lacIPOZYA})X74$ <i>galU galK rpsL</i>	Busby <i>et al.</i> (1983)
M182 Δ crp	M182 <i>crp</i> mutant	Busby <i>et al.</i> (1983)
MC1000	$\Delta(\text{lacIPOZYA})X74$ <i>galU galK</i> $\Delta(\text{araABC-leu})$	Spiro & Guest (1987)
MC4100	$\Delta(\arg F-lac)U169$ <i>rpsL</i>	Sawers & Suppmann (1992)
RM3133	MC4100 <i>arcA</i> (Tc)	Sawers & Suppmann (1992)
VJS691	$\Delta(\arg F-lac)U169$ $\Delta(\text{trpEA})2$	Rabin & Stewart (1993)
VJS1357	VJS691 (<i>narXL</i>)235	Rabin & Stewart (1993)
VJS3042	<i>narQ251-Tn10d</i> (Tc)	Rabin & Stewart (1993)
W3110	F ⁻ , λ^-	Our laboratory collection
Plasmids		
pRS415	Reporter plasmid	Simons <i>et al.</i> (1987)
pGS1739	<i>PydhY-lacZ</i> fusion in pRS415 (−189 to +176 relative to transcript start point)	This study
pGS1831	As pGS1739 but with a mutated FNR site as indicated in Fig. 1(b)	This study
pGS2010	As pGS1739 but with a mutated −42 heptamer as indicated in Fig. 1(b)	This study
pGS2011	As pGS1739 but with a mutated −16 heptamer as indicated in Fig. 1(b)	This study
pGS2012	As pGS1739 but with a mutated +6 heptamer as indicated in Fig. 1(b)	This study
pGS2013	As pGS1739 but with a mutated +15 heptamer as indicated in Fig. 1(b)	This study

introduced as single copies into the λ attachment site of isogenic *E. coli* strains (Simons *et al.*, 1987). Lysogeny status was verified using the method of Powell *et al.* (1994).

Site-directed mutagenesis of the FNR site (TTGATAACGATCAA to ATCATAACGATGAT; mutated bases shown in bold type) was achieved using PCR and appropriate synthetic oligonucleotides. Mutagenesis of the four possible NarL/P sites (heptamer −42, TAACGAT to TTA⁺AAAT; heptamer −16, TAATCAC to ATAAATC; heptamer +6, TAACCAT to TTAAGGT; and heptamer +15, AGGATTA to TCAATTT, mutated bases shown in bold type) was also achieved using PCR. DNA sequencing after ligation into pRS415 verified the authenticity of the altered promoters before transfer to the *E. coli* chromosome as single-copy fusions.

For 5' random amplification of cDNA ends (RACE)-PCR transcript mapping, RNA was isolated from anaerobic cultures of *E. coli* MC1000 using Qiagen RNeasy mini kits according to the manufacturer's instructions. The transcript start was identified using 2 μ g *E. coli* RNA per RACE reaction according to the manufacturer's instructions (Roche). The initial cDNA template was generated using oligonucleotide primer RydhY (TCTAATAGTGGAGGATCCACCGGGTTTCATT). The cDNA was polyadenylated and then amplified using RydhY and the oligo(dT) anchor primer (Roche), yielding a

single product of ~400 bp (as judged by agarose gel electrophoresis), which was subjected to DNA sequencing.

The extent of the *ydH* operon was determined using the *Reverse-iT* One-Step RT-PCR kit (ABgene) and total RNA prepared as described above. The oligonucleotides used were: TATACCGCGACACCTGCCG (forward primer *ydH*Y); TGGCAGCACACAATCTACTG (forward primer *ydH*V); GCATCTGTATCGCCGGTTCG (forward primer *ydH*X); ACCGCATAACACACATTTCG (reverse primer *ydH*Y); CTCTTAATATATTACCTGTC (reverse primer *ydH*V); CGAGAGAAACACGGCGCGCG (reverse primer *ydH*W); CCATCCCCAGAA-CAAATTTG (reverse primer *ydH*X); GCCACGAGCAGCAAGCAAGG (reverse primer *ydH*U); CTCCGATACGCATTCTCGC (reverse primer *ydH*T). Control reactions lacking reverse transcriptase used the *ydH*Y forward primer and the *ydH*Y reverse primer.

Gel retardation and footprinting assays. Radiolabelled *ydH*Y promoter fragments (−189 to +176 relative to the transcript start) were prepared by PCRs (50 μ l total volume) with the same primers used to create the *ydH*Y-*lacZ* fusion in the presence of [α -³²P]dATP (0.37 MBq). The amplified product was purified from Tris-acetate-buffered agarose gels (Sambrook & Russell, 2001) using a Qiagen gel extraction kit (Qiagen). The purified labelled promoter DNA was

incubated with 8 μ M FNR-D154A (FNR*), an FNR protein that retains the ability to bind DNA under aerobic conditions (Ziegelhoffer & Kiley, 1995), together with Tris-HCl, pH 8.0 (20 mM), glycerol (5%, v/v), KCl (100 mM), BSA (0.1 mg ml⁻¹), DTT (1 mM) and calf thymus DNA (3 μ g) for 5 min, before separating the FNR*:DNA complexes from DNA by electrophoresis in polyacrylamide gels buffered with Tris/borate/EDTA (TBE) buffer (90 mM Tris, 90 mM borate, 2 mM EDTA). After electrophoresis the gels were transferred to filter paper (3MM, Whatman) and dried for autoradiography. For gel retardation assays with maltose binding protein (MBP)-NarL (0–4 μ M) and MBP-NarP (0–4 μ M), the Nar proteins were phosphorylated by incubation with acetyl phosphate (50 mM) for 45 min at 25 °C. The proteins were then incubated with radiolabelled *ydhY* promoter DNA (as above) in binding buffer: HEPES, pH 8.0 (20 mM), MgCl₂ (5 mM), potassium glutamate (50 mM), DTT (1 mM) and calf thymus DNA (3 μ g), for 15 min before separation of protein–DNA complexes on 6% polyacrylamide gels buffered with TBE. After electrophoresis the gels were transferred to filter paper (3MM, Whatman) and dried for autoradiography. Footprinting reactions with FNR* (2 μ M), phosphorylated MBP-NarL (0–6 μ M) and phosphorylated MBP-NarP (0–6 μ M) were done essentially as described by Darwin *et al.* (1997) with *PydhY* (–189 to +176) used to construct pGSI739 as the target DNA (see above). The promoter fragment was radiolabelled using Klenow fragment and an appropriate α [³²P]dNTP at either the *Bam*HI site (NarP) or the *Eco*RI site (NarL). The MBP-NarL and MBP-NarP proteins were purified as previously described (Darwin *et al.*, 1997).

Construction of a *ydhY*–*T* mutant. A disruption in the *ydhYVWXUT* operon of W3110 was obtained by linear transformation based on the method of Yu *et al.* (2000). Oligonucleotides containing 3' sequences complementary to the first or last 20 bp of the chloramphenicol-resistance cassette of plasmid pACYC184 (Martinez *et al.*, 1988) and 5'-end sequences flanking *ydhY*–*T* were constructed. Linear DNA carrying the resistance cassette and flanking regions was generated by PCR. *E. coli* strain W3110 containing the plasmid pTP223 (Tet^R) (Potete & Fenton, 1984), which carries the λ *red* recombinase genes under the control of an IPTG-inducible promoter, were grown overnight at 37 °C and diluted (1:100) in L broth containing tetracycline (25 μ g ml⁻¹) and IPTG (2 mM) and grown to OD₆₀₀ ~0.3. Electrocompetent cells were prepared and transformed with ~5 μ g PCR product then incubated in 1 ml L broth at 37 °C for 1 h before plating on selective medium. The resulting colonies (Cm^R) were immediately cured of pTP223 and the presence of the mutation was confirmed by PCR and DNA sequencing. Further transfer of the *ydhY* mutation into clean genetic backgrounds was achieved using bacteriophage P1_{vir}-mediated transduction (Sambrook & Russell, 2001).

Biolog Phenotype Microarray (PM) studies. Cultures of *E. coli* W3110 and the isogenic *ydhY* mutant (JRG5199) were grown for 16 h at 37 °C on R2A agar (Biolog) under anaerobic conditions. Colonies were picked from the plate and suspended in 15 ml IF-0 inoculating fluid (Biolog) to a density that matched the Biolog 85% turbidity standard (OD₄₂₀ ~0.12). The Biolog PM1 and PM2 phenotyping arrays, which contain different carbon sources, were directly inoculated with 100 μ l bacterial suspension per well. For PM3 and PM4 (nitrogen, phosphorus and sulphur sources) the bacterial suspensions were supplemented with glycerol (20 mM) and ferric citrate (2 μ M). All arrays were incubated anaerobically at 37 °C in a Labsystems iEMS Reader and were monitored hourly for 24 h by measuring the increase in OD₅₉₀ due to reduction of the redox indicator tetrazolium violet. All fluids, standards and PM plates were purchased from Biolog. Data were collected from two independent screens in which the differences between the tetrazolium violet reduction rates under each condition were less than 10%.

RESULTS

FNR binds at a site located 42.5 bp upstream of the *ydhY* transcript start

Earlier studies have shown that *ydhY* expression is FNR-dependent *in vivo* and *in vitro*, and that the size of the *in vitro* transcription product is consistent with the presence of an FNR-dependent class II promoter (Kang *et al.* 2005), but the precise transcript start site has not been mapped. Here, RACE-PCR was used to identify the *ydhY* transcript start site (Fig. 1a), which placed the centre of the FNR site at –42.5, a class II location (Fig. 1b). Evidence for direct interaction of FNR with the proposed FNR binding site upstream of *ydhY* was sought using gel retardation assays. To simplify the experiments, an FNR protein (FNR-D154A; referred to as FNR*) that retains some DNA binding activity in the presence of air was used in the gel retardation assays (Ziegelhoffer & Kiley, 1995). These showed that FNR* was able to bind to the unaltered *PydhY* but not to the same promoter fragment carrying mutations in the predicted FNR site (Fig. 1c). Furthermore, mutation of the FNR site in a plasmid-based *ydhY*–*lacZ* fusion abolished FNR-mediated regulation (Fig. 1d), and binding of FNR* at the FNR site in the *ydhY* promoter was confirmed by DNase I footprinting (Fig. 1e). Thus, the data presented are entirely consistent with the findings of Kang *et al.* (2005).

The *ydhY* gene is part of a larger transcriptional unit, the *ydhYVWXUT* operon

Inspection of genes adjacent to *ydhY* and the results of transcript profiling (Kang *et al.*, 2005) raised the possibility that *ydhY* is the first gene of a six-gene operon (*ydhYVWXUT*). The lengths (in nucleotides) of the intergenic regions are: 20 *ydhY*–*ydhV*; 3 *ydhV*–*ydhW*; 12 *ydhW*–*ydhX*; –4 *ydhX*–*ydhU*; and 3 *ydhU*–*ydhT* (Fig. 2). Therefore, RT-PCR using RNA isolated from anaerobic cultures of *E. coli* was used to determine the approximate downstream limits of the *ydhY* transcript. Using pairs of primers in adjacent genes it was established that *ydhY* and *ydhV* are co-transcribed, as are *ydhV*, *W* and *X*, and *ydhX*, *U* and *T* (Fig. 2). This shows that the *ydhY*–*T* genes are co-expressed from the FNR-dependent class II promoter upstream of *ydhY*.

Expression of *ydhY* is repressed by nitrate and nitrite

The possibility that the *ydhY*–*T* operon might be involved in anaerobic respiration was investigated by measuring *ydhY* promoter activity in the presence of alternative electron acceptors. Cultures of JRG5146 (*E. coli* MC1000 λ *ydhY*–*lacZ*) were grown in the presence of fumarate, DMSO, trimethylamine-*N*-oxide, nitrate or nitrite. In the presence of glucose, anaerobic expression of *ydhY*–*T* was approximately twofold lower than that of cultures lacking

Fig. 2. Organization of genes in the *ydhY* operon. The positions and polarities of ORFs (open arrows) and RT-PCR products (double-headed filled arrows) are shown. The numbers above the brackets indicate the number of nucleotides separating the coding regions. Total RNA from anaerobic cultures of *E. coli* MC1000 was used as the template for RT-PCR using appropriate oligonucleotide primers. The ethidium bromide-stained gel shows the RT-PCR products indicated in the diagram. Con, control reaction lacking reverse transcriptase using the *ydhY* forward and reverse primers; M, standard molecular mass markers. The predicted and observed sizes of the RT-PCR products are indicated.

Fig. 1. Expression of *ydhY* is controlled by a class II FNR-dependent promoter. (a) DNA sequence data obtained from RACE-PCR. The DNA sequence of the anchor primer is shown in bold type; the sequence of the *ydhY* transcript is underlined. (b) Nucleotide sequence of the *ydhY* promoter. The transcript start is the upper-case C marked +1. The consensus FNR site centred at -42.5 is boxed and the core inverted repeat is highlighted in bold type. Four potential NarL binding sites are indicated by arrows (matches to the consensus are underlined) and labelled according to the system adopted by Darwin *et al.* (1997). Thus, heptamer -42 is centred 42 bp upstream of the transcript start, heptamer -16 is centred 16 bp upstream of the transcript start, and two heptamers (centred at +6 and +15) are arranged as a 7-2-7 heptamer pair. The DNA sequences of the mutated FNR site and Nar heptamers are shown below the main sequence. (c) Binding of FNR to the *ydhY* promoter. Radiolabelled *ydhY* promoter fragments [native promoter, lanes 1 and 2; promoter with the FNR site mutated (TTGATAACGATCAA→ATCATAACGATGAT), lanes 3 and 4] were incubated with (+) or without (-) 8 μ M FNR* (FNR-D154A) for 10 min before separating FNR*-DNA complexes (C) from free DNA (F) in TBE-buffered polyacrylamide gels and subsequent visualization by autoradiography. (d) Anaerobic activation of *ydhY* expression is abolished by mutation of the consensus FNR site. Anaerobic cultures of MC1000 (*lac*) or JRG1728 (*fmr lac*) containing pGS1739 (*ydhY-lacZ*) or pGS1831 (*ydhY-lacZ* with the FNR site inactivated as above) were grown at 37 °C in L broth to OD₆₀₀ 0.4–0.6. Promoter activities were determined by measuring β -galactosidase activities. Values are means $\pm$ SD of triplicate assays from three independent cultures. (e) FNR* DNase I footprint at the *ydhY* promoter. The *ydhY* promoter was radiolabelled on the top strand. The reaction in the track labelled '-' contained no FNR* protein; the reaction in the track labelled FNR contained FNR* (2 μ M dimer). The solid line below the panel indicates the region of protection. The gel was calibrated with a Maxam and Gilbert G track (G).

glucose (Table 2). Furthermore, β -galactosidase activity from the single-copy *ydhY-lacZ* fusion under aerobic conditions in the absence of FNR was increased approximately twofold compared with the parent, confirming the observations made with the multi-copy plasmid-based reporter (Fig. 1d). The mechanism of this apparent FNR-mediated repression under aerobic conditions is unknown at this time. Addition of fumarate did not significantly change *ydhY-T* expression under anaerobic conditions. However, supplementation of the medium with DMSO or trimethylamine *N*-oxide enhanced anaerobic *ydhY-T* expression, whereas anaerobic expression was significantly lower in the presence of nitrate or nitrite (Table 2).

The response to nitrate and nitrite was partially abolished in the *narXL* and *narPQ* mutants and was completely abolished in a *narXL narPQ* double mutant (Table 3), suggesting a role for both two-component systems in the regulation of *ydhY-T* expression. This pattern of regulation, along with the predicted functions of the encoded proteins, is consistent with the suggestion that the *ydhY-T* operon is associated with anaerobic respiration, perhaps by

Table 2. *In vivo* transcription of *ydhY* in the presence of alternative electron acceptors

Cultures of MC1000 λ *ydhY-lacZ* (JRG5146) and JRG1728 (*fnr*) λ *ydhY-lacZ* (JRG5147) monolysogens were grown at 37 °C in L broth containing the indicated supplements to OD₆₀₀ 0.4–0.6. Promoter activities were determined by measuring β -galactosidase activities. Values are means $\pm$ SD of triplicate assays from three independent cultures.

Strain and growth conditions	Promoter activity (Miller units)
JRG5146, aerobic	45 $\pm$ 2
JRG5146, anaerobic	1760 $\pm$ 168
JRG5146, aerobic plus glucose (0.4 %)	48 $\pm$ 11
JRG5146, anaerobic plus glucose (0.4 %)	842 $\pm$ 78
JRG5147 (<i>fnr</i>), aerobic	89 $\pm$ 6
JRG5147 (<i>fnr</i>), anaerobic	147 $\pm$ 12
JRG5146 (parental), anaerobic plus fumarate (40 mM)	2010 $\pm$ 171
JRG5146 (parental), anaerobic plus DMSO (40 mM)	2500 $\pm$ 163
JRG5146 (parental), anaerobic plus trimethylamine <i>N</i> -oxide (40 mM)	3620 $\pm$ 195
JRG5146 (parental), anaerobic plus nitrate (40 mM)	133 $\pm$ 8
JRG5146 (parental), anaerobic plus nitrite (5 mM)	957 $\pm$ 107

contributing to the maintenance of redox balance in the presence of specific electron acceptors.

Analysis of the *ydhY-T* promoter region revealed the presence of four potential heptameric NarL/P binding sites resembling the consensus TACYMT. These are all appropriately located to repress *ydhY-T* operon expression (Fig. 1b). One heptamer, centred at –42 (heptamer –42), matches the consensus (indicated by underlining) at five positions (TAACGAT), and is in the middle of the FNR site (centred at –42.5), and thus is located in a similar position to a NarL heptamer (centred at –50), which overlaps the FNR site in the *nrfA* promoter (Darwin *et al.*, 1997). A second heptamer (heptamer –16) that also matches the consensus at five positions is centred at –16 (TAATCAC). The two remaining heptamers (heptamers +6 and +15) both match the consensus at six positions and are arranged as a 7-2-7 inverted repeat centred at +10.5

Table 3. Effect of lesions in the NarPQ and NarXL two-component sensor-regulators on *ydhY* expression

Cultures of strains with the indicated genotype carrying the single-copy λ *ydhY-lacZ* fusion were grown at 37 °C under anaerobic conditions in L broth (–) or L broth containing nitrate (40 mM) or nitrite (5 mM) to OD₆₀₀ 0.4–0.6. Promoter activities were determined by measuring β -galactosidase activities. Values are means $\pm$ SD of triplicate assays from three independent cultures.

Genotype	β -Galactosidase activity (Miller units)		
	–	+ Nitrate	+ Nitrite
Parental (VJS691; Table 1)	1976 $\pm$ 132	136 $\pm$ 8	949 $\pm$ 97
<i>narXL</i>	1908 $\pm$ 161	542 $\pm$ 23	958 $\pm$ 38
<i>narPQ</i>	1980 $\pm$ 164	125 $\pm$ 30	1281 $\pm$ 62
<i>narXLPQ</i>	1750 $\pm$ 102	1764 $\pm$ 70	1758 $\pm$ 91

(TAACCATaaAGGATTA). Whereas NarL can potentially recognize all these heptamers, NarP only interacts with heptamers in a 7-2-7 arrangement (Darwin *et al.*, 1997). DNase I footprinting confirmed that NarP recognized the predicted 7-2-7 site (Fig. 3a). The MBP–NarP footprint covered ~33 bp (–10 to +23) punctuated by two hypersensitive sites (+6 and +7), and thus the NarP footprint at the *ydhY-T* promoter is similar to that observed at *nirB* (Darwin *et al.*, 1997). Like NarP, NarL clearly protected the 7-2-7 region, but in contrast to NarP, the NarL footprint extended further upstream to –29 and thus encompassed the –16 heptamer, with hypersensitive sites at +19 and –15 (Fig. 3b). There was no obvious protection across the –42 heptamer; although the intensity of the DNA fragments in this region appeared weaker, no clear boundaries could be defined. This might be due to the –42 heptamer being a poorer match to the Nar consensus than the 7-2-7 site. The extended region of protection observed with NarL compared to NarP was consistent with the presence of only one retarded NarP–*ydhY* complex in gel retardation assays, whereas with NarL, multiple complexes were resolved (Fig. 3c). The PydhY–NarP complex also appeared to be more stable than the PydhY–NarL complexes in the gel retardation assay (as evidenced by the smeared appearance of the bands corresponding to the NarL complexes).

To study the contribution of the NarL/P sites to the regulation of *ydhY-T* expression, each was inactivated in turn by site-directed mutagenesis. The individual heptamers were altered by base-pair replacements designed to disrupt the sites, whilst maintaining other important promoter elements (Table 1). The altered promoter regions were fused to *lacZ* in the reporter plasmid pRS415 and transferred to the chromosome of *E. coli* MC1000 (*lac*). After growth of *E. coli* MC1000 strains containing the various promoter mutants at 37 °C to OD₆₀₀ 0.4–0.6 under anaerobic conditions in L broth or L broth containing

Fig. 3. DNase I footprint and gel retardation analyses for the *ydH* promoter. (a) Protection by NarP. The concentrations of MBP-NarP protein, which had been pre-incubated with 50 mM acetyl phosphate for 45 min to generate active MBP-NarP, in the reactions are shown above each lane. (b) Protection by NarL. The concentrations of MBP-NarL protein, which had been phosphorylated as in (a), in the reactions are shown above each lane. Regions of protection are indicated by solid lines and hypersensitive sites are marked by arrows. The location of the 7-2-7 site is marked by the convergent arrows. The gels were calibrated by Maxam and Gilbert G tracks (M). (c) Binding of NarL and NarP to the *ydH* promoter. Radiolabelled *PydH* DNA was incubated with the indicated amounts of MBP-NarL or MBP-NarP before separation of protein-DNA complexes by gel electrophoresis. The unbound *PydH* DNA and the *PydH*-Nar complexes are indicated.

nitrate (40 mM) or nitrite (5 mM), promoter activities were determined by measuring β -galactosidase. The results (Table 4) confirmed the response of the *ydH-T* promoter to the presence of nitrate and nitrite, as described above. Mutation of the -42 heptamer (overlapping the FNR site) impaired repression in the presence of nitrate, but not nitrite (Table 4), despite the absence of clear protection of the -42 heptamer by phospho-NarL in the footprinting assay. Mutation of the upstream element (+6) of the 7-2-7 site impaired repression by both nitrate and nitrite, showing that this site is important for both nitrate- and nitrite-mediated repression (Table 4). However, mutation of the downstream element (+15) of the 7-2-7 site resulted in a promoter with lower activity than that of the unaltered

promoter in the absence of nitrate or nitrite. Repression by nitrate was unaffected by mutation of the +15 heptamer (Table 4). Although nitrite was a less effective repressor when the +15 heptamer was mutated, the effect was not as obvious as when the +6 heptamer of the 7-2-7 site was disrupted. A plausible explanation for these observations is that mutation of the +6 or +15 heptamers inhibits phospho-NarP binding, allowing nitrite-activated phospho-NarL to bind at the remaining unaltered heptamer (either at the +6 heptamer, where NarL acts as an effective repressor, or at the +15 heptamer, where NarL does not contribute significantly to repression). In this way the effect on nitrite-mediated repression of mutating the +6 heptamer will be more apparent than mutation of the +15 heptamer. Mutation of the -16 heptamer did not affect repression by nitrate, despite the protection afforded to this region of the promoter by NarL (Fig. 3b). However, mutation of the -16 heptamer enhanced repression in the presence of nitrite, suggesting that NarL binding at -16 antagonizes NarP action at the 7-2-7 site. Overall, the simplest explanation for the results obtained is that the 7-2-7 site is involved in nitrite repression, the -42 heptamer and the upstream element of the 7-2-7 site contribute to nitrate repression, and the -16 site modulates the response to nitrite. This is consistent with the known DNA binding properties of NarP (recognizes 7-2-7 sites) and NarL (recognizes both 7-2-7 sites and isolated heptamers) (Darwin *et al.*, 1997).

Further studies showed that *ydH-T* expression was not affected in *crp*, *arcA*, *oxyR* and *soxRS* mutants (results not shown). Thus, it is proposed that in the presence of nitrate or nitrite NarL/P repress *ydH* expression by promoter occlusion.

A *ydH-T* mutant does not exhibit an anaerobic growth defect

Anaerobic batch cultures of *E. coli* W3110 and two independent *ydH-T* operon mutants exhibited the same growth rates and growth yields when L broth, L broth plus glucose (0.4%), or Evans minimal medium plus glucose (20 mM) was used as the medium (results not shown). Furthermore, measurement of final OD₆₀₀ of cultures of W3110 and the *ydH-T* mutant grown in L broth plus glucose (0.4%) supplemented with nitrate (40 mM) or nitrite (5 mM) indicated that the growth of the mutant was not impaired in the presence of these alternative electron acceptors (not shown). Biolog PM phenotyping arrays use the reduction of a tetrazolium dye to integrate respiration of bacteria during an experiment (Bochner, 2003). Ninety-six-well plates containing a different medium in each well are inoculated and a colour change resulting from the reduction of the redox dye is used to monitor respiratory activity of the culture. This showed that the *ydH-T* mutant was not significantly impaired on any of the carbon, nitrogen or phosphorous sources tested. However, reduction of the tetrazolium dye was lower for the *ydH-T*

Table 4. Effect of mutation of the predicted NarL/P binding sites within the *ydhY* promoter

Cultures of MC1000 containing the indicated single-copy *ydhY* promoter mutants fused to *lacZ* were grown at 37 °C under anaerobic conditions in L broth (–), or L broth containing nitrate (40 mM) or nitrite (5 mM) to OD₆₀₀ 0.4–0.6 before promoter activities were determined by measuring β -galactosidase. The unaltered *ydhY* promoter region (–189 to +176) contained all four potential NarL/P binding sites: heptamer –42, heptamer –16, heptamer +6 and heptamer +15; the +6 and +15 heptamers are arranged in the 7-2-7 pattern recognized by NarP. The DNA sequences of the sites and the mutations introduced to inactivate them are shown in Fig. 1(b). Values are means $\pm$ SD of triplicate assays from three independent cultures. The ratios of promoter activity in the presence of nitrate or nitrite to that observed in the absence of nitrate and nitrite are shown in parentheses.

Supplement	Mutation				
	Unaltered	–42 mutant	–16 mutant	+6 mutant	+15 mutant
–	1760 $\pm$ 168	1529 $\pm$ 89	1517 $\pm$ 62	1622 $\pm$ 89	1482 $\pm$ 122
Nitrate	133 $\pm$ 8 (0.08)	605 $\pm$ 25 (0.40)	148 $\pm$ 9 (0.10)	786 $\pm$ 129 (0.48)	150 $\pm$ 23 (0.10)
Nitrite	957 $\pm$ 107 (0.54)	855 $\pm$ 95 (0.56)	682 $\pm$ 56 (0.45)	1213 $\pm$ 60 (0.75)	988 $\pm$ 109 (0.66)

mutant when several compounds (L-cysteine, D-cysteine, L-cysteic acid, hypotaurine and butane sulphonic acid) were supplied as the sole source of sulphur, but these differences were not apparent when measured as final OD₆₀₀ readings in simple anaerobic growth tests in the presence of L-cysteine or D-cysteine in glucose–Evans minimal medium (results not shown). It has been shown elsewhere that *E. coli* is able to utilize tetrahydrothiophene 1-oxide (THTO) as an electron acceptor under anaerobic conditions. However, the genes required were not identified (Meganathan & Schrementi, 1987). Growth of the *ydhY*–*T* mutant JRG5199 in Evans minimal medium supplemented with 0.1 % casamino acids and either 0.3 % (w/v) glucose or 0.5 % (w/v) glycerol as carbon source in the presence of THTO (25 mM) was similar to that of the parental W3110 strain (results not shown). Similar results were obtained when 1 mM tungsten (possibly required for YdhV activity; see above) was supplied in place of molybdenum (not shown). Thus, it was concluded that although the *ydhY*–*T* operon appears to play a role related to anaerobic metabolism of some sulphur compounds, this does not include THTO utilization under the conditions tested.

DISCUSSION

The experiments reported here show that the *ydhY* gene is the first gene in a six-gene operon (*ydhY*–*T*) that encodes a predicted oxidoreductase. The substrate used by the system has not been identified, but the phenotypic characterization of the *ydhY*–*T* mutant revealed respiratory defects in the utilization of five sulphur sources under anaerobic conditions. However, further biochemical investigations will be required to determine the exact role of the six gene products of the operon, and to define the exact nature of the substrate(s) used by the Ydh system. If the *ydhY*–*T* operon proves to be involved in anaerobic sulphur metabolism, then this operon represents a possible example of non-orthologous gene displacement (Koonin *et al.*, 1996), because the equivalent chromosomal location in *Salmonella enterica* serovar Typhimurium is occupied by

the *ttr* operon, encoding tetrathionate reductase (Hensel *et al.*, 1997).

Consistent with the hypothesis that YdhY–*T* has a role in anaerobic metabolism, gel retardation, footprinting, site-directed mutagenesis and transcript-mapping experiments showed that the oxygen-responsive transcription factor FNR directly activates transcription of *ydhY*–*T* under anaerobic conditions by binding at a site located at position –42.5 relative to the transcript start, confirming and extending earlier work that shows that *ydhY* is expressed from a class II FNR-dependent promoter (Kang *et al.*, 2005). Furthermore, *ydhY*–*T* expression is regulated by the NarXL and NarPQ nitrate- and nitrite-responsive two-component systems, confirming the results of previous transcription-profiling studies (Constantinidou *et al.*, 2006).

DNA sequence analysis identified four potential Nar binding heptamers (Fig. 1b). Footprinting assays showed that NarP recognized the 7-2-7 site centred at +10.5 and that NarL recognized a larger region of the *ydhY*–*T* promoter that included the 7-2-7 site and –16 heptamer. No clear protection of the –42 heptamer by NarL was detected. However, site-directed mutagenesis indicated that the –16 heptamer and the +15 heptamer, although protected in the *in vitro* footprinting assays, were not essential for nitrate-responsive *ydhY*–*T* expression *in vivo*, whereas both the –42 and the +6 heptamer made significant contributions, despite the absence of strong protection by NarL of the –42 heptamer in DNase I footprints. This suggests that *in vivo* there are other factors that lead to occupation of the –42 heptamer by NarL in the presence of nitrate. The –42 heptamer is located in the middle of the FNR site and the overlap of the FNR and NarL sites suggests that *in vivo* these proteins compete for occupation of this region of the promoter, with FNR acting positively and NarL negatively to control *ydhY*–*T* expression. This simple mechanism should ensure that *ydhY*–*T* is maximally expressed in the absence of both oxygen and nitrate. In contrast to NarL, the nitrite-responsive NarP

protein recognizes heptamers organized as 7-2-7 inverted repeats (Darwin *et al.*, 1997), such as heptamers +6 and +15. Inactivation of either the +6 or +15 heptamer affected *ydhY-T* expression in the presence of nitrite, suggesting that NarP acts negatively at this site, presumably by competing with RNA polymerase. The enhanced repression observed in the presence of nitrite when the -16 heptamer was mutated suggests that the presence of NarL at the -16 site inhibits the action of NarP at the 7-2-7 site. Detailed *in vitro* analysis of NarL/NarP-*ydhY* promoter interactions will be necessary to further investigate this possibility. Mutation of the +6 heptamer site also impaired nitrate-mediated repression, suggesting that NarL also inhibits RNA polymerase binding by occluding the transcription start point. Thus, it appears that NarL and NarP adopt overlapping mechanisms to inhibit *ydhY-T* expression. Both compete with RNA polymerase for occupation of the *ydhY* promoter in the region of the transcript start, but in addition, NarL competes with FNR to inhibit FNR-mediated anaerobic activation of *ydhY-T* expression. This additional feature, i.e. NarL is capable of occupying more of the *ydhY-T* promoter than NarP, could explain why NarL appears to be more effective than NarP in inhibiting *ydhY-T* expression. NarL-mediated repression of the *frdA* promoter is achieved by NarL binding over a large region centred near the transcription start site and including the FNR site (Li *et al.*, 1994). Similarly, at the FNR-activated NarL-repressed *dmsA* promoter NarL protects a large region that includes the sites for both FNR and RNA polymerase binding (Bearson *et al.*, 2002). Thus, NarL-mediated repression of the *ydhY* promoter follows the general pattern established by the *frdA* and *dmsA* promoters.

Whilst several NarL-repressed FNR-dependent promoters have been studied, FNR-dependent promoters that are repressed by NarP are uncommon because NarP tends to act as an activator, although recent transcript-profiling experiments suggest that NarP might have a wider role as a repressor than previously thought (Constantinidou *et al.*, 2006). The transcript-profiling experiments reveal that a number of transcripts are less abundant in cultures of the *narXL* mutant grown in the presence of nitrate (Constantinidou *et al.*, 2006). Examination of a *narXL narP* double mutant indicates that the absence of NarP enhances the abundance of transcripts from 37 operons, mainly associated with hydrogen and dicarboxylate metabolism (Constantinidou *et al.*, 2006). Like *ydhY-T*, several of the operons suggested to be NarP-repressed, on the basis of transcript profiling, possess credible matches to the NarP consensus site close to the transcript start site, consistent with NarP-mediated repression (Constantinidou *et al.*, 2006). NarP has been reported to act as a negative regulator of the FNR-dependent class II *fdnGHI* promoter under low-nitrate conditions (Wang & Gunsalus, 2003), although this promoter has also been shown to be weakly activated by NarP (Rabin & Stewart, 1993). Nevertheless, in contrast to the *ydhY-T* promoter, where NarP-mediated

repression requires a 7-2-7 site centred at +10.5, occupation of which will probably inhibit RNA polymerase binding, the *fdnGHI* promoter possesses a NarP-binding 7-2-7 site centred at -104.5 that is thought to antagonize activation by NarL (Wang & Gunsalus, 2003). Thus, the architecture of the *ydhY-T* promoter appears to be somewhat different from that of previously characterized FNR-, NarL- and NarP-regulated promoters, having evolved to be repressed in the presence of nitrate and nitrite by using NarP/NarL to inhibit RNA polymerase binding, and NarL to inhibit FNR binding. The relative degrees of repression of the *ydhY-lacZ* fusion in the presence of nitrite (approximately twofold) or nitrate (~13-fold) suggest that NarL-mediated inhibition of FNR recruitment is a key component of the regulation of the *ydhY-T* promoter, and that NarP-mediated inhibition of RNA polymerase binding is partially overcome by the ability of FNR to recruit RNA polymerase.

Whilst it is clear that FNR, NarL and NarP control expression of *ydhY-T*, there are likely to be additional regulatory features. For example, expression is lowered in the presence of glucose (Table 2), and it is possible that a specific regulator that responds to the presence of the YdhY-T substrate is required for full activation. Identification of the substrate for the YdhY-T-catalysed reaction is thus an important goal for future work.

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REFERENCES

- Bearson, S. M. D., Albrecht, J. A. & Gunsalus, R. P. (2002). Oxygen and nitrate-dependent regulation of *dmsABC* operon expression in *Escherichia coli*: sites for FNR and NarL protein interactions. *BMC Microbiol* **2**, 13.
- Berks, B. C., Palmer, T. & Sargent, F. (2005). Protein targeting by the bacterial twin arginine translocation (Tat) pathway. *Curr Opin Microbiol* **8**, 174–181.
- Bochner, B. R. (2003). New technologies to assess genotype-phenotype relationships. *Nat Rev Genet* **4**, 309–314.
- Browning, D., Lee, D., Green, J. & Busby, S. (2003). Secrets of bacterial transcription initiation taught by the *Escherichia coli* FNR protein. In *Signals, Switches, Regulons and Cascades: Control of Bacterial Gene Expression*, pp. 127–142. Edited by D. A. Hodgson & C. M. Thomas. Cambridge, UK: Cambridge University Press.
- Busby, S., Kotlarz, D. & Buc, H. (1983). Deletion mutagenesis of the *Escherichia coli* galactose operon promoter region. *J Mol Biol* **167**, 259–274.
- Constantinidou, C., Hobman, J. L., Griffiths, L., Patel, M. D., Penn, C. W., Cole, J. A. & Overton, T. W. (2006). A reassessment of the FNR

regulon and transcriptomic analysis of the effects of nitrate, nitrite, NarXL and NarPQ as *Escherichia coli* adapts from aerobic to anaerobic growth. *J Biol Chem* **281**, 4802–4815.

Darwin, A. J. & Stewart, V. (1996). The Nar modulon systems: nitrate and nitrite regulation of anaerobic gene expression. In *Regulation of Gene Expression in Escherichia coli*, pp. 343–359. Edited by E. C. C. Lin & A. S. Lynch. Austin, TX: R. G. Landes.

Darwin, A. J., Tyson, K. L., Busby, S. J. W. & Stewart, V. (1997). Differential regulation by the homologous response regulators NarL and NarP of *Escherichia coli* K-12 depends on DNA binding site arrangement. *Mol Microbiol* **25**, 583–595.

Eiglmeier, K., Honore, N., Iuchi, S., Lin, E. C. C. & Cole, S. T. (1989). Molecular genetic analysis of FNR-dependent promoters. *Mol Microbiol* **3**, 869–878.

Evans, C. G. T., Herbert, D. & Tempest, D. W. (1970). The continuous cultivation of micro-organisms. 2. Construction of a chemostat. *Methods Microbiol* **2**, 277–327.

Greenberg, J. T., Monach, P., Chou, J. H., Josephy, D. & Demple, B. (1990). Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc Natl Acad Sci U S A* **87**, 6181–6185.

Guest, J. R., Green, J., Irvine, A. S. & Spiro, S. (1996). The FNR modulon and FNR-regulated gene expression. In *Regulation of Gene Expression in Escherichia coli*, pp. 317–342. Edited by E. C. C. Lin & A. S. Lynch. Austin, TX: R. G. Landes.

Heinzinger, N. K., Fujimoto, S. Y., Clark, M. A., Moreno, M. S. & Barrett, E. L. (1995). Sequence analysis of the *phs* operon in *Salmonella typhimurium* and the contribution of thiosulfate reduction to anaerobic energy metabolism. *J Bacteriol* **177**, 2813–2820.

Hensel, M., Shea, J. E., Baumler, A. J., Gleeson, C., Blattner, F. & Holden, D. W. (1997). Analysis of the boundaries of *Salmonella* pathogenicity island 2 and the corresponding chromosomal region of *Escherichia coli* K-12. *J Bacteriol* **179**, 1105–1111.

Jervis, A. J. & Green, J. (2007). *In vivo* demonstration of FNR dimers in response to lower O₂ availability. *J Bacteriol* **189**, 2930–2932.

Kang, Y., Weber, K. D., Qiu, Y., Kiley, P. J. & Blattner, F. R. (2005). Genome-wide expression analysis indicates that FNR of *Escherichia coli* K-12 regulates a large number of genes of unknown function. *J Bacteriol* **187**, 1135–1160.

Kiley, P. J. & Beinert, H. (2003). The role of Fe–S proteins in sensing and regulation in bacteria. *Curr Opin Microbiol* **6**, 181–185.

Kletzin, A., Mukund, S., Kelley-Crouse, T. L., Chan, M. K., Rees, D. C. & Adams, M. W. W. (1995). Molecular characterisation of the genes encoding the tungsten-containing aldehyde ferredoxin oxidoreductase from *Pyrococcus furiosus* and formaldehyde ferredoxin oxidoreductase from *Thermococcus litoralis*. *J Bacteriol* **177**, 4817–4819.

Koonin, E. V., Mushegian, A. R. & Bork, P. (1996). Non-orthologous gene displacement. *Trends Genet* **12**, 334–336.

Lazazzera, B. A., Beinert, H., Khoroshilova, N., Kennedy, M. C. & Kiley, P. J. (1996). DNA-binding and dimerization of the Fe–S containing FNR protein from *Escherichia coli* are regulated by oxygen. *J Biol Chem* **271**, 2762–2768.

Lee, A. I., Delgado, A. & Gunsalus, R. P. (1999). Signal-dependent phosphorylation of the membrane-bound NarX two-component sensor-transmitter protein of *Escherichia coli*: nitrate elicits a superior anion ligand response compared to nitrite. *J Bacteriol* **181**, 5309–5316.

Lennox, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190–206.

Li, J., Kustu, S. & Stewart, V. (1994). *In vitro* interaction of nitrate-responsive regulatory protein NarL with DNA target sequences in

fdnG, *narG*, *narK* and *frdA* operon control regions of *Escherichia coli* K-12. *J Mol Biol* **241**, 150–165.

Martinez, E., Bartolome, B. & Delacruz, F. (1988). pACYC184-derived cloning vectors containing the multiple cloning site and *lacZx* reporter gene of pUC8/9 and pUC18/19 plasmids. *Gene* **68**, 159–162.

Meganathan, R. & Schrementi, J. (1987). Tetrahydrothiophene 1-oxide as an electron acceptor for *Escherichia coli*. *J Bacteriol* **169**, 2862–2865.

Miller, J. H. (1972). Assay of β -galactosidase. In *Experiments in Molecular Genetics*, pp. 352–354. Edited by J. H. Miller. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Poteete, A. R. & Fenton, A. C. (1984). λ red-dependent growth and recombination of phage P22. *Virology* **134**, 161–167.

Powell, B. S., Court, D. L., Nakamura, Y., Rivas, M. P. & Turnbough, C. L., Jr (1994). Rapid confirmation of single copy lambda prophage integration by PCR. *Nucleic Acids Res* **22**, 5765–5766.

Rabin, R. S. & Stewart, V. (1992). Either of two functionally redundant sensor proteins, NarX and NarQ, is sufficient for nitrate regulation in *Escherichia coli* K-12. *Proc Natl Acad Sci U S A* **89**, 8419–8423.

Rabin, R. S. & Stewart, V. (1993). Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in *Escherichia coli* K-12. *J Bacteriol* **175**, 3259–3268.

Sambrook, J. W. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Sawers, G. & Suppmann, B. (1992). Anaerobic induction of pyruvate formate-lyase gene expression is mediated by the ArcA and FNR proteins. *J Bacteriol* **174**, 3474–3478.

Simons, R. W., Houman, F. & Kleckner, N. (1987). Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**, 85–96.

Spiro, S. & Guest, J. R. (1987). Regulation and over-expression of the *fur* gene of *Escherichia coli*. *J Gen Microbiol* **133**, 3279–3288.

Stewart, V. (1994). Dual interacting two-component regulatory systems mediate nitrate- and nitrite-regulated gene expression in *Escherichia coli*. *Res Microbiol* **145**, 450–454.

Sutton, V. R., Mettert, E. L., Beinert, H. & Kiley, P. J. (2004). Kinetic analysis of the oxidative conversion of the [4Fe–4S]²⁺ cluster of FNR to a [2Fe–S]²⁺ cluster. *J Bacteriol* **186**, 8018–8025.

Turner, R. J., Papish, A. L. & Sargent, F. (2004). Sequence analysis of bacterial redox enzyme maturation proteins (REMPs). *Can J Microbiol* **50**, 225–238.

Uden, G., Achebach, S., Holighaus, G., Tran, H.-Q., Wackwitz, B. & Zeuner, Y. (2002). Control of FNR function of *Escherichia coli* by O₂ and reducing conditions. *J Mol Microbiol Biotechnol* **4**, 263–268.

Wang, H. & Gunsalus, R. P. (2003). Coordinate regulation of the *Escherichia coli* formate dehydrogenase *fdnGHI* and *fdhF* genes in response to nitrate, nitrite, and formate: roles for NarL and NarP. *J Bacteriol* **185**, 5076–5085.

Williams, S. B. & Stewart, V. (1997). Discrimination between structurally related ligands nitrate and nitrite controls autokinase activity of the NarX transmembrane signal transducer of *Escherichia coli* K-12. *Mol Microbiol* **26**, 911–925.

Wing, H. J., Williams, S. M. & Busby, S. J. W. (1995). Spacing requirements for transcription activation by *Escherichia coli* FNR protein. *J Bacteriol* **177**, 6704–6710.

Yamamoto, K., Hirao, K., Oshima, T., Aiba, H., Utsumi, R. & Ishihama, A. (2005). Functional characterisation *in vitro* of all two-component signal transduction systems from *Escherichia coli*. *J Biol Chem* **280**, 1448–1456.

Yu, D., Ellis, H. M., Lee, E.-C., Jenkins, N. A., Copeland, N. G. & Court, D. L. (2000). An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci U S A* **97**, 5978–5983.

Zheng, M., Doan, B., Schneider, T. D. & Storz, G. (1999). OxyR and SoxRS regulation of *fur*. *J Bacteriol* **181**, 4639–4643.

Ziegelhoffer, E. C. & Kiley, P. J. (1995). *In vitro* analysis of a constitutively active mutant form of the *Escherichia coli* global transcription factor FNR. *J Mol Biol* **245**, 351–361.

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