The *Escherichia coli* *yhjA* gene, encoding a predicted cytochrome *c* peroxidase, is regulated by FNR and OxyR

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The *Escherichia coli* FNR protein is an oxygen-responsive global transcription factor, and OxyR is a key regulator of the peroxide stress response. Here both FNR and OxyR are shown to regulate expression of the *E. coli* *yhjA* gene. The *yhjA* gene encodes a predicted cytochrome *c* peroxidase, a bacterial haem-containing protein involved in the peroxide stress response through its ability to convert hydrogen peroxide to water. It is shown that the *yhjA* gene of *E. coli* possesses a class II FNR site and an OxyR site upstream of the *yhjA* transcript start. Expression of *yhjA* was found to be dependent on this unusual combination of FNR and OxyR under conditions of oxygen starvation. Phenotypic analysis of the *yhjA* mutant revealed increased sensitivity to exogenous hydrogen peroxide and organic peroxides during growth under anaerobic conditions, consistent with the observed regulation and predicted function of the *yhjA* gene product.

INTRODUCTION

The *Escherichia coli* FNR protein is an oxygen-responsive global transcription factor (Constantinidou *et al.*, 2006; Covert *et al.*, 2004; Gonzalez *et al.*, 2003; Kang *et al.*, 2005; Partridge *et al.*, 2006; Salmon *et al.*, 2003). Under anaerobic conditions FNR contains a [4Fe–4S] cluster that promotes dimerization of the protein and enhances site-specific DNA binding (Lazazzera *et al.*, 1996). In the presence of oxygen, the iron–sulphur clusters disassemble, leading to the formation of FNR monomers and subsequent inhibition of DNA binding, thereby switching off FNR-activated genes (Crack *et al.*, 2004, 2006; Kiley & Beinert, 2003; Lazazzera *et al.*, 1996; Sutton *et al.*, 2004a, b). Upon binding of the active protein to DNA, FNR activates transcription by recruiting RNA polymerase, or can alternatively repress transcription by inhibiting the formation of productive promoter–RNA polymerase interactions (reviewed by Browning *et al.*, 2002). Transcription activation is facilitated by the formation of specific protein–protein contacts between the activating regions (AR1, AR2 and AR3) of FNR and regions of RNA polymerase (Bell & Busby, 1994; Blake *et al.*, 2002; Lamberg *et al.*, 2002). The DNA sequence recognized by FNR consists of an inverted repeat with a consensus TTGATnnnnATCAA (Eiglmeier *et al.*, 1989). 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) that permit the formation of only one protein–protein contact, between

AR1 of FNR and the C-terminal domain of the RNA polymerase α -subunit. However, the more common architecture is that of the class II promoters, which have an FNR site centred at, or close to, –41.5 relative to the transcript start (Browning *et al.*, 2002; Guest *et al.*, 1996; Wing *et al.*, 2000). This arrangement allows the formation of multiple protein–protein contacts between RNA polymerase and all three activating regions of FNR (Bell & Busby 1994; Blake *et al.*, 2002; Li *et al.*, 1998; Williams *et al.*, 1997).

The FNR protein primarily coordinates gene regulation in remodelling central metabolism in response to changes in oxygen availability in many bacteria (Guest *et al.*, 1996; Korner *et al.*, 2003), but it also exerts effects over other systems, and new members of the FNR regulon are still being uncovered (Covert *et al.*, 2004; Kang *et al.*, 2005). For example, FNR has been implicated in the peroxide stress response as an anaerobic activator of cytochrome *c* peroxidase (CCP) in a number of bacteria including *Neisseria gonorrhoeae*, *Paracoccus denitrificans* and *Pseudomonas stutzeri* (Turner *et al.*, 2003; van Spanning *et al.*, 1997; Vollack *et al.*, 1999). Furthermore, the FNR homologue, Anr, activates CCP expression in *Pseudomonas aeruginosa* (Zimmermann *et al.*, 1991).

The primary role of CCP is thought to be in peroxide stress resistance, because of its ability to catalyse the conversion of hydrogen peroxide to water (Minard & McAlister-Henn, 2001; Seib *et al.*, 2004, reviewed by Attack & Kelly, 2006). However, because typical bacterial CCP proteins are periplasmic dihaem-containing proteins that use a mono-haem cytochrome *c* as an electron donor they could

Abbreviations: CCP, cytochrome *c* peroxidase; RACE, random amplification of cDNA ends.

potentially allow the use of hydrogen peroxide as a terminal electron acceptor.

Here, a search for new members of the *E. coli* FNR regulon identified the *yhjA* gene as a likely candidate. The expression of this gene, which is predicted to encode a CCP, is shown to be driven from an FNR-dependent class II promoter that is also dependent on OxyR. The phenotype of the *yhjA* mutant indicates a role for YhjA in the peroxide stress response of *E. coli*.

METHODS

Bacterial strains and growth conditions. All the bacterial strains and plasmids used in this work are listed in Table 1. For β -galactosidase assays, L broth (Sambrook & Russell, 2001) containing appropriate antibiotics was inoculated (1:50) from overnight cultures. Cultures were incubated under aerobic (250 ml conical flasks containing 10 ml medium shaken at 250 r.p.m.), micro-aerobic (250 ml conical flasks containing 150 ml medium shaken at 100 r.p.m.), or anaerobic (bottles filled to the neck, without shaking) conditions at 37 °C until an OD₆₀₀ of 0.4–0.6 was reached, at which point β -galactosidase activities were measured (Miller, 1972). Oxygen transfer rates were measured as described by Wainwright *et al.* (2005).

Nucleic acid methods. DNA was isolated and manipulated by conventional methods (Sambrook & Russell, 2001). Promoter regions of interest were amplified from the chromosome of MC1000 by PCR using the following oligonucleotides engineered to contain *EcoRI/BamHI* restriction sites: *yjhB*, JDP1 and JDP2; *yfgF*, JDP3 and 4; *yhjA*, JDP5 and 6; *yecI*, FyecI and RyecI; *smf*, Fsmf and Rsmf (Table 1). The sequence of each fragment was verified by DNA sequencing after ligation into *EcoRI/BamHI*-digested pRS415 (Simons *et al.*, 1987). Selected constructs were then transferred to λ RZ5 (Simons *et al.*, 1987) and the resulting (promoter–*lacZ*) fusions were introduced in single copy into the λ attachment site of isogenic *E. coli* strains. Lysogeny status was verified using the method of Powell *et al.* (1994). Further transfer of the promoter fusions into different genetic backgrounds was achieved using bacteriophage P1_{vir}-mediated transduction (Sambrook & Russell, 2001).

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 *yhjA* transcript start was identified using 2 μ g *E. coli* RNA per RACE reaction according to the manufacturer's instructions (Roche) with oligonucleotides JDP7 and JDP8 (Table 1).

Site-directed mutagenesis of the consensus FNR site (TTGAT-N₄-ATCAA to ATCAT-N₄-ATGAT) and putative OxyR-binding site (ATAGgcacaggCTATcttattgATAGtTtatAttcAT to TATCgcacaggGATActtattgTATCtAtatTttcTA) was achieved using PCR and appropriate synthetic oligonucleotides, with all mutations confirmed by DNA sequencing.

Construction of mutants. The *yhjA* gene of W3110 was disrupted by linear transformation based on the method of Yu *et al.* (2000). Oligonucleotides containing 3'-end 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 *yhjA* 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) (Poteete & 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 an OD₆₀₀ of ~0.3. Electrocompetent cells were prepared and transformed with approximately 5 μ g of PCR product then recovered in 1 ml L broth for 1 h before plating on selective medium (Cm^R). The resulting colonies were immediately cured of pTP223 and mutations were screened by PCR and DNA sequencing. Further transfer of the mutations into clean genetic backgrounds was achieved using bacteriophage P1_{vir}-mediated transduction (Sambrook & Russell, 2001).

The *oxyR*-deficient mutant for use in promoter assays was generated through P1_{vir}-mediated transduction (Sambrook & Russell, 2001) of the mutation from GSO47 (Zheng *et al.*, 1999) to MC1000, to generate JRG5393.

Overexpression and purification of OxyR and FNR*.

Overexpression of OxyR from MV247 was as described by Haagmans & van der Woude (2000) with modifications (Correnti *et al.*, 2002). Solubilization was aided by the addition of 0.1 % Tween 20 to the bacterial pellet and incubation at room temperature for 30 min prior to sonication. Protein was purified from the cell-free extract using a HiTrap heparin column (Amersham) as described previously (Storz *et al.*, 1990) with modifications according to Kullik *et al.* (1995). The final preparations contained ~60 % OxyR as judged by Coomassie-stained SDS-polyacrylamide gels.

The FNR protein FNR-D154A (designated FNR*) retains DNA-binding activity under aerobic conditions (Ziegelhoffer & Kiley, 1995) and was isolated as described by Meng *et al.* (1998).

Gel retardation assays. Radiolabelled promoter fragments (~50 ng) were incubated with 6 μ M FNR* and/or 0.06 μ M OxyR in binding buffer (Correnti *et al.*, 2002) for 30 min before separating the protein–DNA complexes from DNA on 6 % TBE-buffered polyacrylamide gels. After electrophoresis the gels were transferred to filter paper (3MM, Whatman) and dried for autoradiography.

Disc diffusion assays. Soft-top agar (3 ml, 0.65 % agar) was mixed with an aliquot of a culture (200 μ l, OD₆₀₀ ~0.3) of the strain under investigation grown under anaerobic conditions. The mix was poured evenly onto L agar plates and, once set, a sterile antibiotic filter disc (6 mm) was placed in the centre of the plate. The indicated reagents were added to the centre of the disc and the plates were incubated under anaerobic conditions at 37 °C for 16 h before measuring the zone of growth inhibition around the disc.

Killing curve studies. Bacterial cultures were grown to OD₆₀₀ ~0.3 under anaerobic conditions and then challenged with a stress reagent. Aliquots were removed at *t*=0, *t*=20, *t*=40 and *t*=60 min and serially diluted in L broth before being plated onto L agar plates incubated under aerobic conditions. The numbers of viable bacteria were calculated from the numbers of c.f.u. after 16 h growth at 30 °C under aerobic conditions.

RESULTS

Identification of the *yhjA* gene as a member of the FNR regulon

The *E. coli* FNR protein recognizes DNA sequences related to the FNR consensus (TTGATnnnnATCAA) (Eiglmeier *et al.*, 1989). The key features of this inverted repeat are the A–T base pairs at positions +7 and –7 (underlined), which allow discrimination between FNR sites and sites recognized by related proteins, and the G–C base pairs at +5 and –5 (bold), which form a core interaction with the DNA recognition helices of this family of transcription factors

Table 1. Bacterial strains, plasmids and oligonucleotides

Relevant genotype or properties		Source or reference
<i>E. coli</i> strains		
GS047	MC4100 <i>oxyR</i> -Kan	Zheng <i>et al.</i> (1999)
JRG1728	MC1000 $\Delta(\textit{tyrR-fnr-rac-trg})17\text{ zdd-230-Tn9}$	Lab collection
JRG3462	CAG627 pGS771	Meng <i>et al.</i> (1998)
JRG5066	MC1000/ λ RZ5(<i>PyhjA-lacZ</i>) from pGS1634	This work
JRG5067	JRG1728/ λ RZ5(<i>PyhjA-lacZ</i>) from pGS1634	This work
JRG5354	W3110 $\Delta yhjA$	
JRG5393	MC1000 <i>oxyR</i> -Kan	This work
JRG5609	JRG5393/ λ RZ5(<i>PyhjA-lacZ</i>) from pGS1634	This work
JRG5641	MC1000/ λ RZ5(<i>PyhjA''-lacZ</i>) from pGS2018	This work
JRG5694	MC1000/ λ RZ5(<i>PyhjA'-lacZ</i>) from pGS1829	This work
MC1000	$\Delta(\textit{lacIPOZYA})X74\text{ galU galK } \Delta(\textit{araABC-leu})$	Lab collection
MV247	XL-1 Blue pMV107	Haagmans & van der Woude (2000)
W3110	F ⁻ , λ^-	Lab collection
Plasmids		
pACYC184	Source of <i>cat</i> cassette for linear transformation	Martinez <i>et al.</i> (1988)
pGS1633	<i>PyfgF-lacZ</i> fusion in pRS415 (192 bp promoter fragment, -108 to +84 relative to the transcript start), Ap ^R	This work
pGS1634	<i>PyhjA-lacZ</i> fusion in pRS415 (187 bp promoter fragment, -127 to +60 relative to the transcript start), Ap ^R	This work
pGS1740	<i>PyecI-lacZ</i> fusion in pRS415 (399 bp promoter fragment), Ap ^R	This work
pGS1740a	<i>Psmf-lacZ</i> fusion in pRS415 (383 bp promoter fragment), Ap ^R	This work
pGS1828	<i>PyjhB-lacZ</i> fusion in pRS415 (450 bp promoter fragment), Ap ^R	This work
pGS1829	<i>PyhjA-lacZ</i> fusion in pRS415 with the FNR-binding site altered from TTGAT-N ₄ -ATCAA to ATCAT-N ₄ -ATGAT, Ap ^R	This work
pGS2018	<i>PyhjA::lacZ</i> fusion in pRS415 with the OxyR-binding site altered from ATAGGcacaggCTATcttattATAGtTtatAttcAT to TATCgcacaggGATActtattTATCtAtatTttcTA, Ap ^R	This work
pMV107	pZE24 with a 929 bp fragment encoding OxyR	Haagmans & van der Woude (2000)
pRS415	<i>lacYZA</i> -based promoter-fusion vector, Ap ^R	Simons <i>et al.</i> (1987)
pTP223	<i>lam</i> , <i>bet</i> and <i>exo</i> genes of phage λ , under the control of the <i>lac</i> promoter, Tet ^R	Poteete & Fenton (1984)
Oligonucleotides		
JDP1	TTTTGAATCAACTGTAGGTGGGTATC	This work
JDP2	TTTTGGATCCGTGTATTCTAATATAGCCC	This work
JDP3	TTTTGAATTCTGTAATAATGATGAATTAACATG	This work
JDP4	TTTTGGATCCGCAGAATCCCCACATCC	This work
JDP5	TTTGAATCCCCGCAATCATGATATGCC	This work
JDP6	TTTGGATCCCGGTGGTGAGTTATTGTG	This work
JDP7	CGTAATCCATCAACTGTTTCG	This work
JDP8	GCCTAAAACCTTATTATTTTCAC	This work
FyecI	TTTTGAATCTTAATTATCACTGGGCCCATTAATTAACAG	This work
RyecI	TTTTGGATCCAAGCATTCCAGCGGATCCCATACCTAATAT	This work
Fsmf	TTTTGAATCTCTAAAAGCTCTGAATTCARTTAACACTAGC	This work
Rsmf	TTTTGGATCCTTTCTGTATCGAGGATCCTTATCTCCCTGC	This work

(Schultz *et al.*, 1991; Spiro *et al.*, 1990). The *E. coli* K-12 genome sequence was searched for the presence of consensus FNR sites (TTGATNnnnATCAA) using the search pattern tool in the Colibri database (<http://genolist.pasteur.fr/Colibri>). This analysis yielded a total of 21 sites. Eliminating those located within coding regions, and those associated with prophages, left 12 consensus FNR sites, which were all centred within 345 bp of a start codon.

Of the 12 transcriptional units associated with consensus FNR sites, seven (*cydA*, *ndh*, *narK*, *narG*, *nirB*, *ydhY* and *yfiD*) are known to be FNR-regulated (Green *et al.*, 1998; Guest *et al.*, 1996; Kang *et al.*, 2005). The regulation of the remaining five genes (*yecI*, *yfgF*, *yhjA*, *yjhB* and *smf*) had not been investigated. To test whether the FNR sites upstream of *yecI*, *yjhB*, *yfgF*, *yhjA* and *smf* contributed to regulation of these genes, the corresponding promoter regions (e.g.

Table 2. Effects of oxygen availability and FNR on the regulation of *yecI*, *yfgF*, *yjhB*, *yhjA* and *smf*

Cultures of MC1000 (*lac*) and JRG1728 (*lac fnr*) transformed with plasmids pGS1828 (*PyjhB-lacZ*), pGS1633 (*PyfgF-lacZ*), pGS1634 (*PyjhA-lacZ*), pGS1740 (*PyecI-lacZ*), pGS1740a (*Psmf-lacZ*), were grown under aerobic and anaerobic conditions at 37 °C in L broth to OD₆₀₀ 0.4–0.6. Promoter activities were determined by measuring β-galactosidase activities. Values are means±SD from three independent cultures assayed in triplicate.

Promoter	Aerobic		Anaerobic	
	MC1000	JRG1728 (<i>fnr</i>)	MC1000	JRG1728 (<i>fnr</i>)
<i>PyecI</i>	3375 ± 215	3094 ± 245	2295 ± 105	2025 ± 115
<i>PyfgF</i>	4350 ± 430	3527 ± 300	56831 ± 1220	4360 ± 252
<i>PyjhB</i>	2950 ± 73	2805 ± 146	2963 ± 194	2885 ± 186
<i>PyhjA</i>	99 ± 5	75 ± 4	733 ± 14	63 ± 5
<i>Psmf</i>	280 ± 18	250 ± 27	180 ± 9	175 ± 18

PyecI-lacZ) were fused to *lacZ* in the reporter plasmid pRS415 and transferred into MC1000 (*lac*) and JRG1728 (*fnr lac*). Expression of *yecI*, *yjhB* and *smf* was not affected by the presence or absence of FNR under anaerobic conditions (Table 2), although expression of both *yecI* and *smf* was apparently lower in the absence of oxygen. However, expression of *yfgF*, and *yhjA* was enhanced under anaerobic conditions in an FNR-dependent manner (Table 2). Here the results of experiments to further characterize the regulation and function of *yhjA* are presented.

Expression of *yhjA* is driven from a class II FNR-dependent promoter

Preliminary *in vivo* transcriptional analysis using a plasmid-based *yhjA-lacZ* fusion showed FNR-dependent regulation of *yhjA*. To further characterize the mechanism of regulation, the activity of the *yhjA* promoter region fused to *lacZ* was investigated using single-copy chromosomal

fusions. Promoter activity assays in MC1000 (*lac*) revealed a ~32-fold induction under anaerobic compared to aerobic conditions (Table 3). This anaerobic activation was lowered to only ~3-fold in the *fnr* mutant JRG1728 (*fnr lac*) (Table 3). Therefore, evidence for direct regulation by FNR 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 (Ziegelhoffer & Kiley, 1995). The assays showed that FNR* was able to bind at the native *yhjA* promoter region, but not at the same promoter fragment with mutations in the predicted FNR site (Fig. 1, lanes 2 and 3). Furthermore, mutation of the FNR site essentially abolished FNR-mediated regulation (Table 3).

To investigate the architecture of the *yhjA* promoter, 5' RACE-PCR was used to identify the transcript start. This placed the centre of the FNR site at -41.5 (Fig. 2). Thus, it was concluded that database searching had identified the *yhjA* gene as a member of the FNR regulon with an FNR-dependent class II promoter.

Activation of *yhjA* is co-dependent on the OxyR system

Further inspection of the *yhjA* promoter region identified a potential binding site for the oxidized (disulphide) form of the transcriptional regulator OxyR that matched the consensus (**ATAGnTnnnAnCTATnnnnnnnATAGnTnnnAnCTAT**) in 16 (shown in bold) of 20 bases (Toledano *et al.*, 1994). The 3' end of the potential OxyR site is located only 8 bp upstream of the 5' end of the FNR site (Fig. 2). The OxyR protein acts as a transcriptional regulator initiating, directly or indirectly, the synthesis of more than 40 gene products to counter the effects of peroxide stress, although transcription of some of these genes is also altered during exposure to heat shock or redox-cycling agents (Greenberg & Demple, 1989; Storz & Zheng, 2000; Walkup & Kogoma, 1989; Zheng *et al.*, 2001).

Table 3. Regulation of *yhjA*

Isogenic *E. coli* MC1000 derivatives harbouring chromosomal promoter-*lacZ* fusions (either *yhjA-lacZ*, or *yhjA-lacZ* with a mutated FNR site, from pGS1829, or *yhjA-lacZ* with a mutated OxyR site, from pGS2018) were grown under aerobic, micro-aerobic and anaerobic conditions at 37 °C in L broth to OD₆₀₀ 0.4–0.6. Promoter activities were determined by measuring β-galactosidase activities. Values are means±SD from three independent cultures assayed in triplicate. ND, not done.

Strain	β-Galactosidase activity (Miller units)		
	Aerobic	Micro-aerobic	Anaerobic
MC1000 (<i>yhjA-lacZ</i>)	0.8 ± 0.2	13.3 ± 3.0	31.0 ± 2
JRG1728 <i>fnr</i> (<i>yhjA-lacZ</i>)	0.7 ± 0.1	3.5 ± 0.2	2.0 ± 0.1
JRG5393 <i>oxyR</i> (<i>yhjA-lacZ</i>)	2.0 ± 0.2	5.9 ± 0.7	4.0 ± 0.3
MC1000 (<i>yhjA-lacZ</i> FNR site inactivated)	0.8 ± 0.1	ND	1.1 ± 0.1
MC1000 (<i>yhjA-lacZ</i> OxyR site inactivated)	2.1 ± 0.2	ND	5.6 ± 0.2

To investigate whether OxyR contributes to the regulation of *yhjA*, the *PyhjA-lacZ* fusion was transferred to an *oxyR* mutant. The anaerobic induction of *yhjA* expression observed previously was significantly lowered (Table 3). Furthermore, mutation of the OxyR site essentially abolished OxyR-mediated regulation (Table 3). These data suggest that OxyR is required for expression of *yhjA*. Therefore, evidence for direct regulation of *yhjA* expression was sought using gel retardation assays. These showed that, like FNR, OxyR was able to bind at the unaltered *yhjA* promoter region, but not at the same promoter fragment carrying mutations in the predicted OxyR site (Fig. 1, lanes 4 and 5). Gel retardation assays in which the *yhjA* promoter was incubated with increasing amounts of OxyR revealed an apparent K_d of ~ 10 nM for OxyR binding (not shown). Moreover, in gel retardation assays in which both FNR* and OxyR were present, the migration of the labelled *yhjA* promoter was slower than in the presence of either FNR* or OxyR alone, suggesting that both proteins occupy the *yhjA* promoter simultaneously (Fig. 1, lane 6).

Thus, the regulation of *yhjA* is interesting because transcription appears to be dependent on two transcription factors that respond to seemingly incompatible signals, i.e. lack of oxygen and peroxide stress. Nevertheless, during micro-aerobic growth oxidative stress might be encountered

whilst some FNR activity is retained. However, *yhjA* expression was only partially activated under micro-aerobic (150 ml culture volumes shaken at 100 r.p.m., equivalent to an oxygen transfer rate of $12 \mu\text{mol min}^{-1} \text{ l}^{-1}$) compared to anaerobic conditions (Table 3), indicating that these particular micro-aerobic conditions are not optimal for *yhjA* expression.

A *yhjA* mutant exhibits increased sensitivity to exogenous peroxides

The gene encoding the predicted CCP of *E. coli*, *yhjA*, was expected to contribute to the peroxide stress response. Therefore the sensitivity of a *yhjA* mutant to hydrogen peroxide and organic peroxides during anaerobic growth was tested. Initial disc diffusion assays indicated that the mutant was more sensitive than the parent to hydrogen peroxide, *t*-butyl hydroperoxide and cumene hydroperoxide (not shown). This enhanced sensitivity was confirmed in killing assays using cultures grown under anaerobic conditions to $\text{OD}_{600} \sim 0.3$ before exposure to the peroxide stress reagents for a total of 20, 40 and 60 min (Fig. 3). Complementation of the *yhjA* peroxide-sensitive phenotype by supplying a wild-type copy of *yhjA* *in trans* was not possible, because all attempts to create plasmids that expressed the YhjA protein in an active form were unsuccessful (not shown). Therefore, to ensure that the observed phenotype was caused by disruption of *yhjA* and not by point mutations located elsewhere in the genome, the lesion was transferred by P1 transduction to a clean genetic background, and the observations reported above were confirmed with the new mutant, although this approach does not exclude the possibility of polar effects on neighbouring genes.

Exposure of the *yhjA* mutant to nitrosative stress reagents (sodium nitroprusside, *S*-nitrosoglutathione, 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine, 3-[2-hydroxy-1-methyl-2-nitrosohydrazino]-*N*-methyl-1-propanamine) and the antimicrobial compound sodium hypochlorite in both disc diffusion and killing curve assays

Fig. 1. Interaction between FNR* and OxyR at the *yhjA* promoter. Radiolabelled unaltered *yhjA* promoter (lanes 1, 2, 4 and 6) and corresponding promoter fragments with mutations in the predicted FNR-binding site (lane 3) or the predicted OxyR-binding site (lane 5) were incubated with FNR* (6 μM), OxyR (60 nM), or FNR* and OxyR for 30 min at 25 °C before separation of protein–DNA complexes by electrophoresis. Lane 1, no protein; lanes 2 and 3, FNR*; lanes 4 and 5, OxyR; lane 6, FNR* and OxyR. The positions of free DNA (*yhjA*), *yhjA*–protein complexes and the origin of migration (origin) are indicated.

Fig. 2. Nucleotide sequence of the *yhjA* promoter region. The transcript start (arrow), putative -10 box (italicized), FNR site (boxed), and OxyR site (underlined) are indicated. Matches to the FNR and OxyR consensus sequences are highlighted in bold.

Fig. 3. Sensitivity of the *yhjA* mutant to peroxide stress in liquid culture. Cultures of *E. coli* W3110 (○) and the isogenic *yhjA* mutant (●) were grown to OD₆₀₀ ~0.3 before being treated with the following reagents: (a) hydrogen peroxide, 2 mM; (b) t-butyl hydroperoxide, 0.4 mM; (c) cumene hydroperoxide, 0.2 mM. At *t*=0, 20, 40 and 60 min the number of c.f.u. in the treated cultures was determined. The data presented are the means of triplicate assays from three independent cultures. Error bars (most within the sizes of the symbols) show the standard deviations.

indicated no increased sensitivity compared to the parent strain, suggesting that *yhjA* has a specific role in the peroxide stress response (data not shown).

DISCUSSION

The work presented here indicates that the *yhjA* gene of *E. coli*, which is predicted to encode a cytochrome *c* peroxidase (CCP), plays a role in the peroxide stress response under anaerobic conditions. Inspection of the *E. coli* genome sequence indicates that the *yhjA* gene is a single transcriptional unit. The YhjA protein (465 amino acids) is 46% identical (61% similar over 291 amino acids) to the CCP from *Pseudomonas aeruginosa* (323 amino acids) (Samyn *et al.*, 1995). However, the YhjA protein is somewhat atypical. Generally bacterial CCP proteins possess two haem cofactors, the first (high-potential) site accepts electrons from the donating cytochrome *c* before transferring them to the second (low-potential) site, often referred to as the peroxidatic site, where hydrogen peroxide is reduced to

water (reviewed by Attack & Kelly, 2006). Most physiological and biochemical studies of bacterial CCP proteins have been of these typical dihaem proteins, including those of *Methylococcus capsulatus* (Zahn *et al.*, 1997), *Neisseria gonorrhoeae* (Turner *et al.*, 2003), *Nitrosomonas europaea* (Arciero & Hooper, 1994), *Paracoccus denitrificans* (Gilmour *et al.*, 1993), *Paracoccus pantotrophus* (Goodhew *et al.*, 1990), *Pseudomonas aeruginosa* (Ellfolk & Soininen, 1970; Fülöp *et al.*, 2001), *Pseudomonas nautica* (Alves *et al.*, 1999) and *Rhodobacter capsulatus* (Hanlon *et al.*, 1992). However, the YhjA amino acid sequence reveals the presence of a third haem-binding motif. The additional haem-binding site is located in an N-terminal extension of ~80 amino acid residues, which is absent from the dihaem CCP proteins. The exact role of this extended domain and the third haem is unclear, because the trihaem CCP proteins are poorly characterized. However, it has been suggested that the trihaem CCP proteins may have evolved by fusion of a monohaem cytochrome *c* gene to a dihaem *ccp* gene, consequently bypassing the need for a separate donor system (Attack & Kelly, 2006). Another distinction between CCP proteins is their cellular location, being either soluble periplasmic proteins or anchored to the membrane as lipoproteins. Cellular location is controlled by the presence of N-terminal amino acid sequences that are recognized by the Sec secretion pathway (Braun & Wu, 1993) and either type I or type II signal peptidases, the former generating a soluble periplasmic protein, whilst the latter leads to anchoring as a lipoprotein. Thus, the CCP of *N. gonorrhoeae* possesses a signal peptidase II recognition sequence and is a lipoprotein (Turner *et al.*, 2003). Similarly, YhjA also possesses a potential target for signal peptidase II cleavage (VAIC), suggesting it is likely to be a lipoprotein, perhaps accounting for the lack of success in attempts to create expression plasmids that yielded functional protein.

The peroxide-sensitive phenotype of the *yhjA* mutant reported here is consistent with the assignment of YhjA as a CCP. Whilst bacterial CCP proteins are known to detoxify hydrogen peroxide, there is evidence that they also work with organic peroxides. For example, a *Bacteroides fragilis ccp* mutant exhibits enhanced sensitivity to organic peroxides (t-butyl hydroperoxide and cumene hydroperoxide) compared with hydrogen peroxide (Herren *et al.*, 2003). Likewise, the *E. coli yhjA* mutant was sensitive to organic peroxides and hydrogen peroxide, and the differences in sensitivity to these compounds might reflect the relative contribution of YhjA to their detoxification. Thus, the bioinformatic and phenotypic analyses are consistent with the assignment of the *E. coli* YhjA protein as a trihaem CCP.

The regulation of *yhjA* expression is interesting, because transcription is dependent on two transcription factors, FNR and OxyR, that respond to two seemingly incompatible signals, i.e. oxygen starvation and peroxide stress. It is often under conditions of high aeration that bacteria are exposed to hydrogen peroxide (Seaver & Imlay, 2004), leading to

activation of OxyR. However, these same conditions inactivate FNR (Becker *et al.*, 1996). It is possible that OxyR might be activated by nitrosative stress, as suggested by Kim *et al.* (2002), but analysis of the *yhjA* mutant phenotype did not support this idea. This raises the possibility that both oxidized and reduced OxyR could be functional at the *yhjA* promoter, because both forms of OxyR are able to bind at some promoters. Oxidized OxyR recognizes ATAGnT elements in four adjacent major grooves on one face of the DNA helix (Toledano *et al.*, 1994). In contrast, reduced OxyR contacts ATAGnT elements in two pairs of major grooves separated by one turn of the DNA helix (Toledano *et al.*, 1994). The DNA sequence of the *oxyRS* promoter region is such that both these patterns of binding are possible, and thus OxyR regulates its own expression, when reduced and oxidized, simply by repositioning itself on the DNA. At OxyR-activated promoters, such as *ahpC*, *dps*, *gorA* and *katG*, the ATAGnT elements are arranged such that only oxidized OxyR binds with significant affinity (Toledano *et al.*, 1994). The *yhjA* OxyR site resembles those of OxyR-activated promoters, lacking an additional ATAGnT element. Moreover, the fourth OxyR contact point in *yhjA* does not obviously resemble both ATAGnT and AnCTAT elements, which is necessary to allow redox-state-responsive repositioning of OxyR at *oxyRS* (Toledano *et al.*, 1994). Furthermore, because the *yhjA* OxyR and FNR sites are so close, any additional downstream OxyR contacts will overlap the FNR site and are likely to prevent FNR binding. Thus, the architecture of the *yhjA* promoter, the phenotype of the *yhjA* mutant and the predicted function of the YhjA protein suggest that transcription from the *yhjA* promoter is likely to be restricted to specific environmental conditions in which the bacteria are exposed to peroxides, but oxygen is limited such that some FNR activity is retained. The presence of a consensus FNR site in the *yhjA* promoter is consistent with this idea, because a consensus site is likely to be occupied in preference to other sites when the amount of active FNR is limited. The ability of FNR, located in a typical class II position, to only weakly activate expression of *yhjA* in the absence of OxyR suggests that the role of OxyR might be to counteract the effects of an as-yet-unidentified regulator that inhibits the action of FNR. The extensive mutagenesis of the promoter to inactivate the OxyR site is likely to have disrupted other regulatory elements in this region (−94 to −57), but this was not sufficient to enhance FNR-mediated *yhjA* expression (Table 3), and suggests that any such regulatory element is located upstream (−127 to −94) of the OxyR site.

The regulatory combination of OxyR and FNR might not be restricted to the *yhjA* promoter. The *katG* gene encodes hydroperoxidase I, which acts as a dual-function catalase and peroxidase with a proven role in the peroxide stress response. Expression of *katG* is activated by OxyR binding at a site centred at −52 relative to the transcript start. In addition, there are two credible FNR sites located at −96.5 and −112.5, and although there is no direct evidence of FNR

binding to the *katG* promoter, transcript profiling suggests that FNR activates *katG* expression (Constantinidou *et al.*, 2006). If the *katG* FNR site(s) are functional, then although the *katG* promoter architecture is different to that of *yhjA*, where the FNR and OxyR sites are very close together, the seemingly incompatible combination of FNR and OxyR might be used to control expression of at least two genes associated with the peroxide stress response in *E. coli*.

In conclusion, it has been shown that the YhjA protein is likely to be a CCP that contributes to the survival of *E. coli* upon exposure to peroxide stress, and that expression of the *yhjA* gene is driven from an FNR-dependent class II promoter that also requires OxyR for activity. This unusual use of FNR and OxyR to activate expression suggests that *yhjA* plays a specific role in countering oxidative stress in *E. coli*, possibly in particular micro-aerobic environments. Further extensive *in vitro* analysis is required to determine the exact mechanism by which two regulators that respond to such disparate signals combine to activate *yhjA* expression.

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REFERENCES

- Alves, T., Besson, S., Duarte, L. C., Pettigrew, G. W., Girio, F. M. F., Devreese, B., Vandenberghe, I., Van Beeumen, J., Fauque, G. & Moura, I. (1999). A cytochrome *c* peroxidase from *Pseudomonas nautica* 617 active at high ionic strength: expression, purification and characterization. *Biochim Biophys Acta* **1434**, 248–259.
- Arciero, D. & Hooper, A. (1994). A di-heme cytochrome *c* peroxidase from *Nitrosomonas europaea* catalytically active in both the oxidized and half-reduced states. *J Biol Chem* **269**, 11878–11886.
- Atack, J. M. & Kelly, D. J. (2006). Structure, mechanism and physiological roles of bacterial cytochrome *c* peroxidases. *Adv Microb Physiol* **52**, 73–106.
- Becker, S., Holighaus, G., Gabrielczyk, T. & Uden, G. (1996). O₂ as the regulatory signal for FNR-dependent gene expression in *Escherichia coli*. *J Bacteriol* **178**, 4515–4521.
- Bell, A. & Busby, S. J. W. (1994). Location and orientation of an activating region in the *Escherichia coli* transcription factor FNR. *Mol Microbiol* **11**, 383–390.
- Blake, T., Barnard, A., Busby, S. J. W. & Green, J. (2002). Transcription activation by FNR: evidence for a functional Activating Region 2. *J Bacteriol* **184**, 5855–5861.
- Braun, V. & Wu, H. C. (1993). Lipoproteins: structure, function, biosynthesis and model for protein export. In *New Comprehensive Biochemistry*, vol. 27, *Bacterial Cell Wall*, pp. 319–341. Edited by J.-M. Ghuyssen & R. Hachenbecks. Amsterdam: Elsevier.
- Browning, D. F., Lee, D., Green, J. & Busby, S. J. W. (2002). Secrets of bacterial transcription taught by the *Escherichia coli* FNR protein. In *Signals, Switches, Regulons and Cascades*, pp. 127–142. Edited by

D. Hodgson & C. Thomas. Cambridge, UK: Cambridge University Press.

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 NarQP as *Escherichia coli* K-12 adapts from aerobic to anaerobic growth. *J Biol Chem* **281**, 4802–4815.

Correnti, J., Munster, V., Chan, T. & van der Woude, M. (2002). Dam-dependent phase variation of Ag43 in *Escherichia coli* is altered in a *seqA* mutant. *Mol Microbiol* **44**, 521–532.

Covert, M. W., Knight, E. M., Reed, J. L., Herrgard, M. J. & Palsson, B. O. (2004). Integrating high-throughput and computational data elucidates bacterial networks. *Nature* **429**, 92–96.

Crack, J., Green, J. & Thomson, A. J. (2004). Mechanism of oxygen sensing by the bacterial transcription factor fumarate-nitrate reduction (FNR). *J Biol Chem* **279**, 9278–9286.

Crack, J., Green, J., Le Brun, N. E. & Thomson, A. J. (2006). Detection of sulphide release from the oxygen-sensing [4Fe-4S] cluster of FNR. *J Biol Chem* **281**, 18909–18913.

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.

Ellfolk, N. & Soininen, R. (1970). *Pseudomonas* cytochrome *c* peroxidase. I. Purification procedure. *Acta Chem Scand* **24**, 2126–2136.

Fülöp, V., Watmough, J. & Ferguson, S. J. (2001). Structure and enzymology of two bacterial di-heme enzymes: cytochrome *cd*₁ nitrite reductase and cytochrome *c* peroxidase. *Adv Inorg Chem* **51**, 163–204.

Gilmour, R., Goodhew, C. F., Pettigrew, G. W., Prazeres, S., Moura, I. & Moura, J. J. (1993). Spectroscopic characterization of cytochrome *c* peroxidase from *Paracoccus denitrificans*. *Biochem J* **294**, 745–752.

Gonzalez, R., Tao, H., Purvis, J. E., York, S. W., Shanmugam, K. T. & Ingram, L. O. (2003). Gene array-based identification of changes that contribute to ethanol tolerance in ethanologenic *Escherichia coli*: comparison of KO11 (parent) to LY01 (resistant mutant). *Biotechnol Prog* **19**, 612–623.

Goodhew, C. F., Wilson, I. B., Hunter, D. J. & Pettigrew, G. W. (1990). The cellular location and specificity of bacterial cytochrome *c* peroxidases. *Biochem J* **271**, 707–712.

Green, J., Baldwin, M. & Richardson, J. (1998). Downregulation of *Escherichia coli* *yfiD* expression by FNR occupying a site at –93.5 involves the AR1-containing face of FNR. *Mol Microbiol* **29**, 1113–1123.

Greenberg, J. T. & Dimple, B. (1989). A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. *J Bacteriol* **171**, 3933–3939.

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

Haagmans, W. & van der Woude, M. (2000). Phase variation of Ag43 in *Escherichia coli*: Dam-dependent methylation abrogates OxyR binding and OxyR-mediated repression of transcription. *Mol Microbiol* **35**, 877–887.

Hanlon, S. P., Holt, R. A. & McEwan, A. G. (1992). The 44 kDa *c*-type cytochrome induced in *Rhodobacter capsulatus* during growth with dimethylsulphoxide as an electron acceptor is cytochrome *c* peroxidase. *FEMS Microbiol Lett* **97**, 283–288.

Herren, C. D., Rocha, E. R. & Smith, C. J. (2003). Genetic analysis of an important oxidative stress locus in the anaerobe *Bacteroides fragilis*. *Gene* **316**, 167–175.

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.

Kim, S. O., Merchant, K., Nudelman, R., Bayer, W. F., Keng, T., DeAngelo, J., Hausladen, A. & Stamler, J. S. (2002). OxyR: a molecular code for redox-mediated signalling. *Cell* **109**, 383–396.

Korner, H., Sofia, H. J. & Zumft, W. G. (2003). Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol Rev* **27**, 559–592.

Kullik, I., Toledano, M. B., Tartaglia, L. A. & Storz, G. (1995). Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. *J Bacteriol* **177**, 1275–1284.

Lamberg, K. E., Luther, C., Weber, K. D. & Kiley, P. J. (2002). Characterization of activating Region 3 from *Escherichia coli* FNR. *J Mol Biol* **315**, 275–283.

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.

Li, B., Wing, H., Lee, D., Wu, H. & Busby, S. (1998). Transcription activation by *Escherichia coli* FNR protein: similarities to and differences from the CRP paradigm. *Nucleic Acids Res* **26**, 2075–2081.

Martinez, E., Bartolome, B. & Delacruz, F. (1988). pACYC184-derived cloning vectors containing the multiple cloning site and *lacZ* alpha reporter gene of pUC8/9 and pUC18/19 plasmids. *Gene* **15**, 159–162.

Meng, W., Green, J. & Guest, J. R. (1998). FNR-dependent repression of *ndh* gene expression requires two upstream FNR-binding sites. *Microbiology* **143**, 1521–1532.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Minard, K. I. & McAlister-Henn, L. (2001). Antioxidant function of cytosolic sources of NADPH in yeast. *Free Radic Biol Med* **31**, 832–843.

Partridge, J. D., Scott, C., Tang, Y., Poole, R. K. & Green, J. (2006). *Escherichia coli* transcriptome dynamics during the transition from anaerobic to aerobic conditions. *J Biol Chem* **281**, 27806–27815.

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.

Salmon, K., Hung, S. P., Mekjian, K., Baldi, P., Hatfield, G. W. & Gunsalus, R. P. (2003). Global gene expression profiling in *Escherichia coli* K12: the effects of oxygen availability and FNR. *J Biol Chem* **278**, 29837–29855.

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

Samyn, B., Van Craenenbroeck, K., De Smet, L., Vandenberghe, I., Pettigrew, G. & Van Beeumen, J. (1995). A reinvestigation of the covalent structure of *Pseudomonas aeruginosa* cytochrome *c* peroxidase. *FEBS Lett* **377**, 145–149.

Schultz, S. C., Shields, G. C. & Steitz, T. A. (1991). Crystal-structure of a CAP-DNA complex: the DNA is bent by 90°. *Science* **253**, 1001–1007.

Seaver, L. C. & Imlay, J. A. (2004). Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? *J Biol Chem* **279**, 48742–48750.

- Seib, K. L., Tseng, H. J., McEwan, A. G., Apicella, M. A. & Jennings, M. P. (2004). Defenses against oxidative stress in *Neisseria gonorrhoeae* and *Neisseria meningitidis*: distinctive systems for different lifestyles. *J Infect Dis* **190**, 136–147.
- 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., Gaston, K. L., Bell, A. I., Roberts, R. E., Busby, S. J. & Guest, J. R. (1990). Interconversion of the DNA binding specificities of two related transcription regulators, CRP and FNR. *Mol Microbiol* **4**, 1831–1838.
- Storz, G. & Zheng, M. (2000). Oxidative stress. In *Bacterial Stress Responses*, pp. 47–59. Edited by G. Storz & R. Hengge-Aronis. Washington, DC: American Society for Microbiology.
- Storz, G., Tartaglia, L. A. & Ames, B. N. (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* **248**, 189–194.
- Sutton, V. R., Mettert, E. L., Beinert, H. & Kiley, P. J. (2004a). Kinetic analysis of the oxidative conversion of the $[4\text{Fe-4S}]^{2+}$ cluster of FNR to a $[2\text{Fe-2S}]^{2+}$ cluster. *J Bacteriol* **186**, 8018–8025.
- Sutton, V. R., Stubna, A., Patschkowski, T., Münck, E., Beinert, H. & Kiley, P. J. (2004b). Superoxide destroys the $[2\text{Fe-2S}]^{2+}$ cluster of FNR from *Escherichia coli*. *Biochemistry* **43**, 791–798.
- Toledano, M. B., Kullik, I., Trinh, F., Baird, P. T., Schneider, D. T. & Storz, G. (1994). Redox-dependent shift of OxyR–DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. *Cell* **78**, 897–909.
- Turner, S., Reid, E., Smith, H. & Cole, J. (2003). A novel cytochrome *c* peroxidase from *Neisseria gonorrhoeae*: a lipoprotein from a Gram-negative bacterium. *Biochem J* **373**, 865–873.
- van Spanning, R. J., De Boer, A. P., Reijnders, W. N., Westerhoff, H. V., Stouthamer, A. H. & Van Der Oost, J. (1997). FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation. *Mol Microbiol* **23**, 893–907.
- Vollack, K.-U., Hartig, E., Korner, H. & Zumft, W. G. (1999). Multiple transcription factors of the FNR family in denitrifying *Pseudomonas stutzeri*: characterization of four *fnr*-like genes, regulatory responses and cognate metabolic processes. *Mol Microbiol* **31**, 1681–1694.
- Wainwright, L. M., Elvers, K. T., Park, S. F. & Poole, R. K. (2005). A truncated haemoglobin implicated in oxygen metabolism by the microaerophilic food-borne pathogen *Campylobacter jejuni*. *Microbiology* **151**, 4079–4091.
- Walkup, L. K. B. & Kogoma, T. (1989). *Escherichia coli* proteins inducible by oxidative stress mediated by the superoxide radical. *J Bacteriol* **171**, 1476–1484.
- Williams, S. M., Savery, N. J., Busby, S. J. W. & Wing, H. J. (1997). Transcription activation at Class I FNR-dependent promoters: identification of the activating surface of FNR and the corresponding contact site in the C-terminal domain of the RNA polymerase α subunit. *Nucleic Acids Res* **25**, 1112–1118.
- 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.
- Wing, H. J., Green, J., Guest, J. R. & Busby, S. J. W. (2000). Role of activating region 1 of *Escherichia coli* FNR protein in transcription activation at class II promoters. *J Biol Chem* **275**, 29061–29065.
- Yu, D., Ellis, H. M., Lee, E., 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.
- Zahn, J. A., Arciero, D. M., Hooper, A. B., Coats, J. R. & DiSpirito, A. A. (1997). Cytochrome *c* peroxidase from *Methylococcus capsulatus* Bath. *Arch Microbiol* **168**, 362–372.
- Zheng, M., Doan, B., Schneider, T. D. & Storz, G. (1999). OxyR and SoxRS regulation of *fur*. *J Bacteriol* **181**, 4639–4643.
- Zheng, M., Wang, X., Templeton, L. J., Smulski, D. R., LaRossa, R. A. & Storz, G. (2001). DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J Bacteriol* **183**, 4562–4570.
- 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.
- Zimmermann, A., Reimann, C., Galimand, M. & Haas, D. (1991). Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with FNR of *Escherichia coli*. *Mol Microbiol* **5**, 1483–1490.

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