The *soxRS* response of *Escherichia coli* can be induced in the absence of oxidative stress and oxygen by modulation of NADPH content

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The *soxRS* regulon protects *Escherichia coli* cells against superoxide and nitric oxide. Oxidation of the SoxR sensor, a [2Fe–2S]-containing transcriptional regulator, triggers the response, but the nature of the cellular signal sensed by SoxR is still a matter of debate. *In vivo*, the sensor is maintained in a reduced, inactive state by the activities of SoxR reductases, which employ NADPH as an electron donor. The hypothesis that NADPH levels affect deployment of the *soxRS* response was tested by transforming *E. coli* cells with genes encoding enzymes and proteins that lead to either build-up or depletion of the cellular NADPH pool. Introduction of NADP⁺-reducing enzymes, such as wheat non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase or *E. coli* malic enzyme, led to NADPH accumulation, inhibition of the *soxRS* regulon and enhanced sensitivity to the superoxide propagator methyl viologen (MV). Conversely, expression of pea ferredoxin (Fd), a redox shuttle that can oxidize NADPH via ferredoxin-NADP(H) reductase, resulted in execution of the *soxRS* response in the absence of oxidative stress, and in higher tolerance to MV. Processes that caused NADPH decline, including oxidative stress and Fd activity, correlated with an increase in total (NADP⁺ + NADPH) stocks. SoxS expression can be induced by Fd expression or by MV in anaerobiosis, under conditions in which NADPH is oxidized but no superoxide can be formed. The results indicate that activation of the *soxRS* regulon in *E. coli* cells exposed to superoxide-propagating compounds can be triggered by depletion of the NADPH stock rather than accumulation of superoxide itself. They also suggest that bacteria need to finely regulate homeostasis of the NADP(H) pool to enable proper deployment of this defensive response.

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INTRODUCTION

Reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) and the superoxide and hydroxyl radicals, can be formed as byproducts of respiration and photosynthesis, or as a consequence of enzyme auto-oxidation and oxidase activities (Apel & Hirt, 2004). They are able to react with a wide range of biomolecules, including proteins, lipids and nucleic acids, leading to cell stasis and death (Imlay, 2008). Aerobes have evolved a multi-tasked assortment of protective devices to prevent ROS formation, scavenge ROS once generated, and repair the damage (Imlay, 2008). Whenever ROS build-up overcomes the cellular defence barriers, the outcome is oxidative stress.

The protective responses to ROS exposure have been most thoroughly investigated in *Escherichia coli*. The bulk of the

defensive resources in this species are grouped into two commonly regulated suites of genes belonging to the *soxRS* and *oxyR* regulons (Pomposiello *et al.*, 2001; Blanchard *et al.*, 2007; Imlay, 2008). The *E. coli* transcriptional activator OxyR induces expression of the *oxyR* regulon in response to peroxide treatment, while the *soxRS* system confers tolerance to superoxide and nitric oxide. The sensor of this regulon is the SoxR protein, a dimeric transcription factor that contains [2Fe–2S] centres (Watanabe *et al.*, 2008). When *E. coli* cells are exposed to superoxide-propagating compounds such as the redox-cycling herbicide methyl viologen (MV), the iron–sulphur cluster of SoxR undergoes univalent oxidation to yield the oxidized, active protein. The only known target of activated SoxR is the *soxS* gene (Pomposiello *et al.*, 2001). Increased SoxS levels, in turn, induce expression of more than 100 genes (Blanchard *et al.*, 2007). Different components of the *soxRS* regulon combat the toxic effects of oxidants at various levels, including ROS scavenging, replacement of sensitive targets by resistant counterparts and damage repair (Imlay, 2008).

Abbreviations: Fd, ferredoxin; FPR, ferredoxin-NADP(H) reductase; G6PDH, glucose-6-phosphate dehydrogenase; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; MV, methyl viologen; ROS, reactive oxygen species.

Despite the wealth of knowledge on the components of the *soxRS* response and their mechanism of action, it is still unclear how the oxidative stress condition is sensed by the SoxR protein. Indeed, it is likely that superoxide is not the only oxidant involved in this process, and maybe not even the most important. Liochev & Fridovich (1992) were first to propose that the inducing signal could be a decrease in NADPH levels caused by NADPH oxidation during the generation of superoxide, although the contention has not been rigorously tested. NADPH could keep SoxR reduced and inactive *in vivo* by the action of at least two different SoxR reductases, both using NADPH as electron donor (Kobayashi & Tagawa, 1999; Koo *et al.*, 2003).

In metabolic terms, NAD⁺ serves primarily for energy production and NADPH as a hydride donor for biosynthetic reactions. In addition, NADPH is the reductant or essential cofactor for several enzymes involved in protective and/or antioxidant activities, including glutathione reductase (GR), thioredoxin reductase, NADPH-dependent alkylhydroperoxidase and nitroreductase (Paterson *et al.*, 2002; Imlay, 2008). Notably, several components of the *soxRS* and *oxyR* systems, such as glucose-6-phosphate dehydrogenase (G6PDH), ferredoxin-NADP(H) reductase (FPR), flavodoxin, GR and hydroperoxidase I are NADPH-linked enzymes or proteins. NADPH formation appears to be stimulated when bacteria are exposed to either superoxide radicals (Pomposiello *et al.*, 2001) or H₂O₂ (Brumaghim *et al.*, 2003), suggesting that its accumulation is essential to cope with oxidative stress. On the other hand, high NADPH levels might have pro-oxidant effects by reducing transition metals to their more toxic states (Brumaghim *et al.*, 2003).

The aim of this study was to investigate how directed alterations in NADPH levels affect both deployment of the *soxRS* response and tolerance to oxidative stress in *E. coli*. It was found that transformation of *E. coli* with genes encoding enzymes that reduce NADP⁺ led to build-up of NADPH content, downregulation of the *soxRS* response and higher sensitivity to MV and H₂O₂. Conversely, expression of an NADPH consumer, chloroplast ferredoxin (Fd), resulted in the opposite behaviour. Moreover, NADPH oxidation by Fd or by the NADPH-diaphorase acceptor MV led to significant SoxS accumulation in anaerobiosis, when no superoxide could be formed. Total (NADP⁺ + NADPH) levels significantly increased in response to NADPH declines, and vice versa. These observations indicate that NADPH homeostasis plays a critical role in proper execution of the *soxRS* response.

METHODS

Plasmid construction. The *gapN* cDNA encoding wheat non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN), present in plasmid pRSETB-GAPN, was PCR-amplified using primers ttgaattcaatgacggggacgggg (forward) and aagctttcagcccatggtgtaggac (reverse) (restriction sites for *Eco*RI and *Hind* III in bold, translation start and stop sites in bold and underlined). The product was cloned in

pGEM-T-easy (Table 1), digested with *Eco*RI and *Hind*III, and ligated to compatible sites of pUC18. The resulting plasmid, pUCGAPN, was transformed into *E. coli* BL21(DE3) codon plus (CP) (Table 1). The recombinant protein was expressed under the control of the *lac* promoter fused in-frame to the first five amino acids of the *lacZ* gene product. The GAPN activity of soluble cell extracts was determined spectrophotometrically according to Bustos & Iglesias (2002).

E. coli malic enzyme was expressed from plasmid pETMAE under control of the strong T7 promoter that drives expression of this gene, using the inducer IPTG. This vector was prepared by cloning the coding region of the *E. coli maeB* gene into *Bam*HI/*Hind*III sites of pET28b as described below. The *maeB* gene present in plasmid pGEMT-MaeB (Bologna *et al.*, 2007) was amplified by PCR using two primers with *Bam*HI/*Hind*III restriction sites. The forward and reverse oligonucleotides were designed to hybridize to positions 1–15 and 2262–2280 of the *maeB* gene, respectively, counting from the translation initiation site. The PCR product was digested and ligated into compatible sites in pET28b. Expression of malic enzyme was verified by activity measurements and Western blotting.

Plasmid pSUFd was prepared by cloning the mature region of pea Fd1 into the *Bam*HI/*Hind*III sites of pSU18. This sequence of 350 bp was amplified by PCR from a cDNA encoding the protein (Catalano Dupuy *et al.*, 2004), using primers gatcttgcggatcctatggcttcttac (forward) and ccacaagcttgatcatcatatcatagcatagcagt (reverse) with specific restriction sites in bold. Fd was expressed with 11 additional amino acids as a result of the N-terminal fusion to β -galactosidase, and under control of the *lac* promoter. Details of pETFd construction are given elsewhere (Catalano Dupuy *et al.*, 2004). From this plasmid, mature pea Fd was expressed as a soluble protein in high yield under control of the T7 promoter and a RBS provided by the pET28a vector.

Antibiotics (Table 1) were used at the following concentrations: ampicillin, 100 μ g ml⁻¹; chloramphenicol, 25 μ g ml⁻¹; kanamycin, 40 μ g ml⁻¹.

Determination of β -galactosidase activity. Fd expression in *E. coli* BL21(DE3) CP transformed with pETFd was driven by T7 RNA polymerase in LB broth containing 60 μ M FeSO₄ and 0.1 mM EDTA. These cells also contained plasmid pTN1530, bearing a *soxS*::*lacZ'* operon fusion (Table 1). Bacterial cultures were incubated for 90 min before IPTG addition, and samples were collected at various times in pre-chilled tubes to measure β -galactosidase activity (Miller, 1992).

Determination of SoxS and FPR expression. Overnight cultures of *E. coli* cells transformed with pUCGAPN, pETMAE, pETFd and the corresponding empty plasmids were diluted (1:100) in 50 ml LB medium supplemented with the corresponding antibiotics and 0.5 mM IPTG. Bacterial suspensions were incubated at 30 °C (pUCGAPN) or 37 °C (pETMAE and pETFd) to OD₆₀₀ 0.6–0.8. Suspensions were split into two equal portions and MV was then added to one of these at a final concentration of 0.5 mM. At the times indicated in the legends to Figs 2, 3 and 4, cells were collected by centrifugation (10 min at 7000 g), resuspended in 50 mM Tris/HCl, pH 8, 1 mM EDTA, 0.1 mM PMSF, disrupted by sonic oscillation (5 times for 6 s each, 20% amplitude), and centrifuged (15 min at 18000 g). Soluble fractions were resolved by SDS-PAGE and transferred to nylon membranes, where SoxS and FPR were detected with specific antisera using secondary antibodies conjugated to alkaline phosphatase.

For experiments performed under anaerobic conditions, overnight aerobic cultures were diluted (1:50) in 12 ml degassed LB broth and incubated at 37 °C for 18 h in a BBL Gas-Pak jar with MV. An anaerobic atmosphere was obtained with the bioMérieux envelope system. Cells were collected on ice and broken by sonic oscillation. Cleared lysates were analysed by SDS-PAGE and immunoblotting.

Table 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics*	Source or reference
Strains		
BL21(DE3) CP	F2 <i>ompT hsdSB</i> ($r_B^- m_B^-$) <i>dcm gal</i> BL21(DE3), Cam ^r , tRNAs <i>argU</i> (AGA, AGG), <i>ileY</i> (AUA), <i>leuW</i> (CUA)	Stratagene
BL21(DE3)	F2 <i>ompT hsdSB</i> ($r_B^- m_B^-$) <i>dcm gal</i> BL21(DE3)	Stratagene
B247	MC4100[λ JW2] λ JW2: Φ [<i>soxS'</i> : <i>lacZ'</i>], Amp ^r /Kan ^r	Wu & Weiss (1992)
Plasmids		
pRSETB-GAPN	pRSETB with the coding region of the <i>Tagapn</i> gene	A. Iglesias, personal gift
pGEM T-easy	Vector for cloning PCR products	Promega
pUC18	Amp ^r	Takara Bio
pUCGAPN	pUC18 with the <i>Tagapn</i> gene under control of the <i>lac</i> promoter	This study
pSU18	Derivative of pACYC containing the <i>lacZ</i> gene of pUC18, Cam ^r	Martínez <i>et al.</i> (1988)
pSUFd	pSU18 with the coding region of mature pea Fd1	This study
pETFd	pET28a carrying the coding region of mature pea Fd1 gene, Kan ^r	Catalano Dupuy <i>et al.</i> (2004)
pET28a, pET28b	Vector with <i>T7</i> promoter and N-terminal His-tag, Kan ^r	Novagen
pETMAE	pET28b carrying the coding region of NADP ⁺ -specific <i>E. coli</i> malic enzyme	This study
pTN1530	pNK1415 Δ <i>soxR soxS'</i> : <i>lacZ</i> , Amp ^r	Nunoshiba <i>et al.</i> (1992)
pETSoxS	pET28a with the <i>soxS</i> gene, used to obtain recombinant SoxS	This study

*Amp^r, Ampicillin resistance; Cam^r, chloramphenicol resistance; Kan^r, kanamycin resistance.

Immunoreactive bands were integrated after scanning using the Gel-Pro Analyser 4.0 software pack (Media Cybernetics).

Susceptibility assays. In the disc diffusion assay, 100 μ l bacterial suspension containing $\sim 10^8$ cells was mixed with 3 ml 0.7% (w/v) warm molten agar and poured onto LB agar plates. After hardening, MV and H₂O₂ solutions were added in 5 μ l aliquots to paper discs (5 mm diameter) placed on the agar surface. Zones of growth inhibition were measured after incubation for 24 h at 30 °C (pUCGAPN) or 37 °C (pETFd).

Determination of NADP⁺ and NADPH concentrations. Cultures of *E. coli* BL21 DE3 CP transformed with pUC18, pUCGAPN, pET28a or pETFd were grown aerobically in LB broth (300 ml in a 1000 ml flask) with the appropriate antibiotics and IPTG. When the cultures reached OD₆₀₀ 0.5, quadruplicate samples of 15 ml were taken ($t=0$) and 0.5 mM MV was added. Samples were withdrawn at 30, 60 and 120 min and frozen at -70 °C. Pyridine nucleotides were estimated by the method of Slater & Sawyer (1962), with the following modifications. Cellular pellets corresponding to 15 ml of culture were resuspended in 200 μ l 0.2 M HCl or in 400 μ l 0.2 M NaOH, and centrifuged at 16 000 *g* for 10 min. Supernatants were heated at 95 °C for 90 s and immediately neutralized with 1 M Tris/HCl pH 8 to pH 5–6 (NADP⁺ determinations), or with 0.2 M HCl to pH 7–8 (NADPH measurements). Known amounts of NADP⁺ and NADPH were added to the lysates prior to the heating step to estimate recovery. The reaction was carried out in 1 ml 25 mM Tris/HCl pH 8, 1 mM glucose 6-phosphate, 100 μ M 2,6-dichlorophenol indophenol, 0.5 mM phenazine methosulphate, 2 units G6PDH ml⁻¹ and 25–50 μ l sample, following the change of absorbance at 600 nm. Concentrations were calculated from a NADP⁺/NADPH standard curve (0–500 pmol).

Protein concentrations were estimated in cleared lysates by a dye-binding method (Sedmak & Grossberg, 1977), using BSA as standard.

Statistical analyses. Statistical differences between treatments and strains were analysed by a two-sided *t*-test.

RESULTS

NADPH accumulation causes downregulation of the *soxRS* response of *E. coli*

We have previously shown that *E. coli* cells overexpressing G6PDH from a multicopy plasmid accumulated higher NADPH levels and displayed a protracted *soxRS* response (Giró *et al.*, 2006). However, G6PDH (encoded by the *zwf* gene) is itself induced during the process (Blanchard *et al.*, 2007), complicating interpretation of the results. To compare the effect of NADPH build-up in a manner that is independent of endogenous regulatory networks, we cloned a wheat gene encoding GAPN (Bustos & Iglesias, 2002) in pUC18 to yield pUCGAPN, and used the resulting plasmid to transform bacteria from the *E. coli* BL21(DE3) CP (Table 1). This enzyme was chosen because no endogenous GAPN activity has been reported in *E. coli* (Iddar *et al.*, 2005).

After IPTG induction, the foreign enzyme accumulated in the cytosol of pUCGAPN-transformed cells. Specific GAPN activities increased more than 250-fold in these bacteria, relative to cells harbouring pUC18 [161.0 ± 14.0 vs 0.6 ± 0.2 μ mol (mg protein)⁻¹ min⁻¹]. Expression of GAPN led to a significant increase (from 0.72 to 2.45) in the NADPH:NADP⁺ ratio during the exponential growth phase, accompanied by a 20–25% decrease in total (NADP⁺ + NADPH) contents (Table 2). Exposure of cells transformed with either pUCGAPN or pUC18 to 0.5 mM MV in liquid LB broth had little or no effect on the NADPH/NADP⁺ ratio, which declined less than 20% in both strains. Instead, the treatment led to a time-dependent increase of the total (NADP⁺ + NADPH) pool

Table 2. Effect of MV exposure on NADP(H) levels in *E. coli* cells expressing GAPN or Fd

E. coli BL21(DE3) CP cells harbouring the plasmids indicated were inoculated in LB broth supplemented with the appropriate antibiotics, 0.5 mM IPTG and, in the case of pET28a- and pETFd-transformed bacteria, 60 μ M FeSO₄ and 0.1 mM EDTA. When present, MV concentration was 0.5 mM. Samples were withdrawn after 2 h incubation at 30 °C (pUCGAPN) or 37 °C (pETFd) for determination of NADP⁺, NADPH and protein as described in Methods. Each value represents the mean $\pm$ SEM of three determinations.

Plasmid and treatment	NADPH:NADP ⁺ ratio	(NADPH+NADP ⁺) [nmol (mg protein) ⁻¹]
pUC18	0.72 $\pm$ 0.18	0.94 $\pm$ 0.12
pUC18+MV	0.62 $\pm$ 0.14	2.92 $\pm$ 0.2
pUCGAPN	2.45 $\pm$ 0.52	0.73 $\pm$ 0.15
pUCGAPN+MV	1.96 $\pm$ 0.40	1.68 $\pm$ 0.2
pET28a	0.82 $\pm$ 0.20	1.02 $\pm$ 0.13
pET28+MV	0.65 $\pm$ 0.16	3.01 $\pm$ 0.18
pETFd	0.35 $\pm$ 0.09	1.7 $\pm$ 0.21
pETFd+MV	0.30 $\pm$ 0.06	4.58 $\pm$ 0.2

during the 2 h time-frame of the experiment (Fig. 1). The (NADP⁺+NADPH) build-up followed similar time-courses in the two strains, with GAPN-expressing bacteria displaying lower levels at all times assayed. Cells incubated during the same period in the absence of MV displayed only marginal alterations in total (NADP⁺+NADPH) amounts (Fig. 1), indicating that the increase was caused by the herbicide.

Induction of the *soxRS* response in these MV-treated cells was evaluated by immunoblot analysis of SoxS. In the absence of oxidative stress, SoxS could not be detected, irrespective of GAPN expression (Fig. 2a, lanes 1 and 6). After MV exposure, an immunoreactive band coincident with the molecular mass of SoxS (~12 kDa) was visualized in extracts from both strains, although SoxS was poorly induced in GAPN-expressing bacteria relative to cells transformed with the empty vector (Fig. 2a), as estimated in a number of assays. Evaluation of the SoxS target gene *fpr*, encoding FPR, gave similar results (Fig. 2b), supporting the notion that the *soxRS* regulon was induced to a significantly lower extent in GAPN-expressing high-NADPH cells.

To confirm that the effects observed were caused by alterations of NADPH levels and not by GAPN expression itself or some other consequence of GAPN activity, similar experiments were carried out with BL21(DE3) cells transformed with plasmid pETMAE overexpressing the *E. coli* NADP⁺-specific malic enzyme, which reduces NADP⁺ during the oxidative decarboxylation of malate (Bologna *et al.*, 2007). Exposure of pETMAE-transformed bacteria to MV led to induction of SoxS (Fig. 2c, lanes 5 and 6), but to a lower level than siblings containing the supporting plasmid alone (Fig. 2c, lanes 2 and 3), and even lower than that for GAPN-expressing bacteria (Fig. 2a).

Fig. 1. Accumulation of (NADP⁺+NADPH) in *E. coli* cells exposed to MV. Bacteria transformed with pUC18 or pET28a (●; results identical), pUCGAPN (■) or pETFd (▲) were incubated with 0.5 mM MV in LB broth at 30 °C (pUCGAPN) or 37 °C (pETFd) with vigorous shaking. At the times indicated, aliquots were withdrawn to determine NADP⁺, NADPH and protein contents as described in Methods. The corresponding open symbols indicate samples of each strain that were incubated in the absence of MV. Each data point represents the mean $\pm$ SEM of three determinations.

Expression of a plant ferredoxin induces the *soxRS* response of *E. coli* in the absence of oxidative stress

Since NADPH build-up resulted in inhibition of the *soxRS* response under oxidative stress, we evaluated whether NADPH consumption by an introduced electron acceptor could boost the response when no oxidants were present. Fd is an iron-sulphur protein which can oxidize NADPH to NADP⁺ via FPR (Carrillo & Ceccarelli, 2003). *E. coli* Fd, unlike the isofunctional electron shuttle flavodoxin, does not belong to the *soxRS* regulon (Blanchard *et al.*, 2007). We cloned a pea gene encoding chloroplast Fd1 in the multicopy plasmid pET28a under control of the T7 RNA polymerase promoter (pETFd, Table 1), and used it to transform *E. coli* BL21(DE3) CP that contained a second plasmid (pTN1530) harbouring a *soxS'::lacZ'* fusion. Plant Fd can act as a substrate of *E. coli* FPR (Giró *et al.*, 2006).

The foreign Fd was produced at high yields upon induction of the transformed bacteria with 0.5 mM IPTG, as revealed by SDS-PAGE and immunoblot analysis with specific antisera (Fig. 3a). At these expression levels, iron might become limiting, so the medium was supplemented with 60 μ M FeSO₄ and 0.1 mM EDTA to ensure that the expressed protein was functional as an electron acceptor. Accumulation of an active Fd led to a drop in the NADPH:NADP⁺ ratio relative to cells transformed with pET28a (0.35 vs 0.82), paralleled by a 70 % increase in total (NADP⁺+NADPH) content (Table 2). On exposure

Fig. 2. Expression of enzymes that produce NADPH causes downregulation of the *soxRS* response of *E. coli*. (a) Cells transformed with pUC18 (lanes 1–4) or pUCGAPN (lanes 6–9) were challenged with 0.5 mM MV as described in Methods. Aliquots were taken after 0 (lanes 1 and 6), 30 (lanes 2 and 7), 60 (lanes 3 and 8) and 120 min (lanes 4 and 9) of MV treatment, cells were ruptured by sonic oscillation, and cleared lysates corresponding to 7 µg total soluble protein were analysed by SDS-PAGE and immunoblotting using anti-SoxS antiserum. Lane 5, molecular mass standards. (b) Immunoblots of extracts corresponding to bacteria transformed with pUC18 (lanes 2 and 4) or pUCGAPN (lanes 3 and 5) incubated in the absence (lanes 2 and 3) or in the presence (lanes 4 and 5) of MV for 2 h. Extracts corresponding to 7 µg total soluble protein were analysed by SDS-PAGE and immunoblotting using anti-FPR antiserum. Lane 1, molecular mass markers. (c) Cells transformed with pET28b (lanes 1–3) or pETMAE (lanes 4–6) were challenged with 0.5 mM MV for 0 (lanes 1 and 4), 30 (lanes 2 and 5) and 60 min (lanes 3 and 6) as described in Methods. Samples were processed and analysed with anti-SoxS antiserum as described for (a). The upper parts of panels (a) and (b) show Coomassie brilliant blue-stained portions of the gels as loading controls.

of the cells to 0.5 mM MV, ($\text{NADP}^+ + \text{NADPH}$) levels increased in a time-dependent manner in both strains, with Fd-expressing cells displaying higher values at all times assayed (Fig. 1).

The extent of induction of the *soxRS* response was initially evaluated by measuring transcription from the *soxS'* promoter via β -galactosidase activity. Two hours after addition of IPTG in the absence of MV, β -galactosidase activity had increased approximately eightfold in Fd-expressing cells relative to bacteria containing pET28a alone (4720 vs 585 Miller units), suggesting transcriptional stimulation of the regulon in the absence of oxidative stress.

Activation of the *soxRS* response in Fd-expressing bacteria was confirmed by direct detection of SoxS in immunoblots (Fig. 3b, lane 4). When cells were exposed to MV, SoxS accumulation was higher in Fd-expressing bacteria relative

to siblings transformed with the supporting vector (Fig. 3c). Addition of iron alone had no effect on the levels of SoxS (Fig. 3b, lane 2), indicating that the effect of Fd was due to its electron-accepting activity and not to the establishment of oxidative stress imposed by the transition metal. As anticipated, the SoxS target gene encoding FPR displayed a similar behaviour (Fig. 3d).

Induction of the *soxRS* regulon in anaerobiosis

Although the Fd results reported in the previous section indicate that *soxRS* induction can be accomplished by manipulation of NADPH levels in the absence of oxidants (Fig. 3b, d), the possibility of superoxide generation associated with aerobic metabolism cannot be ruled out in that experimental set-up. We therefore investigated whether the *soxRS* regulon can be induced by Fd expression in anaerobiosis, when superoxide formation is precluded. Fig. 4(a) shows that this was indeed the case. MV is another suitable candidate for depleting the NADPH pool since it is the electron acceptor of four different NADPH-dependent diaphorase activities (Gaudu & Fontecave, 1994; Liochev *et al.*, 1994). The herbicide is reduced by *E. coli* cells in anaerobiosis but cannot engage in redox cycling with oxygen, significantly decreasing its toxicity (Liochev *et al.*, 1994). Addition of MV to anaerobic suspensions of *E. coli* cells led to rapid reduction of the dipyrrolyl compound to its radical form, which could be visualized by appearance of a blue colour in the medium. Under these conditions, SoxS displayed maximal accumulation at 0.5 mM MV (Fig. 4b, lanes 2 and 3). Beyond that concentration, SoxS levels declined (Fig. 4b, lane 4), presumably reflecting increased impairment of the bacterial metabolism. Collectively, the results indicate that the *soxRS* regulon can be activated in anaerobiosis provided that NADPH levels are decreased by suitable electron acceptors such as Fd or MV, although the extent of induction was significantly lower than that attained under aerobiosis (Fig. 4b). These results concur with previous observations that showed anaerobic activation of the *soxRS* regulon by nitric oxide (Nunoshiba *et al.*, 1993), and by diamide, a thiol oxidant expected to deplete the NADPH pool (Privalle *et al.*, 1993). In *Pseudomonas aeruginosa*, phenazines can also activate SoxR anaerobically (Dietrich *et al.*, 2006).

NADPH modulates sensitivity of *E. coli* cells to oxidants

Since NADPH accumulation in the GAPN-expressing cells resulted in a weakened induction of the *soxRS* response, the tolerance of these bacteria to oxidants was analysed by the disc diffusion method. *E. coli* cells containing pUCGAPN were more sensitive to MV than siblings transformed with pUC18 (Fig. 5a). Tolerance to MV was also assayed in *E. coli* B247 cells expressing Fd from the low-copy-number plasmid pSUFd (Table 1). This strain displayed an intrinsically higher sensitivity to MV toxicity as compared with

Fig. 3. NADPH consumption induces the *soxRS* response of *E. coli* cells in the absence of oxidative stress. (a) Fd accumulation in *E. coli* BL21(DE3) CP cells transformed with recombinant plasmid pETFd (Table 1). Bacteria were grown for 2 h after addition of 0.5 mM IPTG at 37 °C in LB broth. Cleared lysates were prepared as described in Methods and analysed by SDS-PAGE and immunoblotting using antiserum raised against pea Fd. Lane 1, purified pea Fd (25 ng); lanes 2 and 3, extracts corresponding to 20 µg total soluble protein from bacteria transformed with pET28a (lane 2) or pETFd (lane 3). (b) Cells were incubated for 2 h in the presence or in the absence of FeSO₄ and IPTG as described in Methods. Lysates corresponding to 12 µg total soluble protein were analysed by SDS-PAGE and immunoblotting using anti-SoxS antiserum. Immunoblots are shown of extracts corresponding to *E. coli* cells challenged with 0.5 mM MV as SoxS-positive control (lane 1); and of bacteria transformed with pET28a supplemented with Fe (lane 2); pETFd plus Fe (lane 3), or pETFd plus Fe and IPTG (lane 4). (c) Cells transformed with pET28a (lanes 1–4) or pETFd (lanes 5–8) were exposed to 0.5 mM MV for 0 (lanes 1 and 5), 30 (lanes 2 and 6), 60 (lanes 3 and 7) or 120 min (lanes 4 and 8) in the presence of 0.5 mM IPTG. Cleared lysates corresponding to 7 µg total soluble protein were loaded onto each lane. Immunoblot analysis with anti-SoxS antiserum was carried out as described above. The graph below the gel shows the amounts of SoxS in arbitrary units (AU; means $\pm$ SEM of three independent determinations), calculated by scanning the immunoblots. Asterisks above each bar represent the statistical significance of the differences among strains: *, $P \leq 0.05$; **, $P \leq 0.001$. (d) FPR immunoblots of extracts corresponding to bacteria transformed with pET28a (lane 1); supplemented with Fe (lane 2); transformed with pETFd supplemented with Fe (lane 3), or with Fe and IPTG

(lane 4). Lane 5, purified FPR (25 ng). The graph below the gel shows the amounts of FPR (mean $\pm$ SEM of two independent determinations), as indicated in (c). **, $P \leq 0.001$.

BL21(DE3) CP (Fig. 5a, b), but tolerance was significantly increased in cells accumulating an active Fd (Fig. 5b).

GAPN-expressing bacteria also displayed enhanced sensitivity to H₂O₂ (Fig. 5c). Manchado *et al.* (2000) have reported that H₂O₂ activates the *soxRS* response at millimolar concentrations like those used in our assay, presumably through depletion of NADPH stocks. Interestingly, H₂O₂ toxicity was diminished in the presence of deferoxamine, an iron chelating agent (Fig. 5c), suggesting that increased sensitivity of GAPN-expressing cells results from both protracted deployment of the *soxRS* response and the pro-oxidant role of NADPH in providing reduced iron for Fenton-type reactions.

DISCUSSION

The results presented herein support an earlier proposal by Liochev & Fridovich (1992), who suggested that the *soxRS* regulon of *E. coli* is able to sense the NADPH status and respond accordingly. Expression of an NADPH-producing

Fig. 4. NADPH depletion induces the *soxRS* response of *E. coli* BL21(DE3) CP in anaerobiosis. (a) Cells transformed with pET28a (lane 1), or with pETFd (lane 2) supplemented with Fe (lane 3) or Fe and IPTG (lane 4) were grown for 18 h in a BBL Gas-Pak jar. Bacteria were ruptured and extracts corresponding to 50 µg soluble protein were analysed by immunoblotting with anti-SoxS antiserum as described in Methods. Lane 6, SoxS immunoblots of extracts corresponding to cells challenged aerobically with 0.5 mM MV as SoxS-positive control. Lane 7, molecular mass standards. (b) Non-transformed cells were exposed for 24 h to the indicated concentrations of MV under the anaerobic conditions described above. Cleared extracts corresponding to 20 µg soluble protein were analysed by SDS-PAGE and immunoblotting with SoxS antiserum. Lanes 1–4, cells were incubated with 0, 0.2, 0.5 and 1 mM MV, respectively; lane 5, exposed to 0.5 mM MV in aerobiosis; lane 6, purified SoxS (25 ng) used as control.

Fig. 5. Susceptibility of *E. coli* cells to MV or H₂O₂ toxicity depends on expression of NADPH producers or consumers. (a) BL21(DE3) CP bacteria transformed with pUC18 or pUCGAPN were grown to exponential phase and then 0.1 ml of the suspension was added to soft agar and poured over LB agar plates. Paper discs were charged with 5 µl of the indicated concentrations of MV. (b) Expression of Fd in *E. coli* B247 cells transformed with pSUFd results in increased tolerance to MV in disc diffusion assays. Experimental conditions were as described for (a). (c) BL21(DE3) CP bacteria transformed with pUC18 or pUCGAPN were grown and exposed to H₂O₂ as indicated in (a). Protection by an iron-chelating agent was demonstrated by using plates supplemented with 10 mM deferoxamine mesylate (Def). Each bar represents the mean ± SEM of three experiments.

enzyme, GAPN, led to increased NADPH:NADP⁺ ratios (Table 2), partially inhibited the induction of SoxR-dependent components (Fig. 2), and resulted in enhanced sensitivity to redox-cycling oxidants (Fig. 5). Conversely, incorporation of an NADPH consumer such as the electron acceptor Fd resulted in lower NADPH:NADP⁺ ratios (Table 2), and enhanced induction of the *soxRS* regulon in the absence of oxidative stress or even of oxygen (Figs 3 and 4). These results agree with previous observations indicating that the *soxRS* response is downregulated by overexpression of G6PDH from a multicopy plasmid (Giró *et al.*, 2006), and induced by expression of NADPH-consuming enzymes and electron acceptors, including FPR, flavodoxin and desulfoferrodoxin (Gaudu *et al.*, 2000; Krapp *et al.*, 2002; Zheng *et al.*, 1999). They also agree with observations made on *E. coli* *zwf* mutants that are chronically NADPH-deficient and display an enhanced *soxRS* response (Liochev & Fridovich, 1992). We therefore propose that the size and degree of reduction of the NADP(H) pool could influence the progress of the *soxRS* response and act as a signal of the cellular redox status for SoxR modulation. This signalling role could be exerted through the SoxR reductase activities, for which NADPH is the electron donor (Kobayashi & Tagawa, 1999; Koo *et al.*, 2003).

We used anaerobic conditions to show that the *soxRS* regulon can be activated in the absence of oxygen, but it is not clear if this activation confers any physiological advantage for cell welfare under anaerobiosis. By inducing G6PDH, it could help to maintain and/or increase NADPH levels when they are depleted by sudden metabolic demands or xenobiotics, but evidence for such a role is still lacking.

The nature of the physiological electron acceptor(s) of SoxR in the absence of oxidative stress remains elusive. The midpoint redox potential of purified *E. coli* SoxR is −290 mV (Ding *et al.*, 1996), indicating that many cellular oxidants could react with SoxR and activate it in non-stressed cells. Since this is not the case, SoxR must be maintained in its reduced state by an active process, presumably mediated by the SoxR reductases. However, binding of SoxR to its target sequence in DNA results in an upward shift of the redox potential to +200 mV (Gorodetsky *et al.*, 2008), therefore limiting activation to strong oxidants. It is at present unclear which of the two species, the soluble or the DNA-bound SoxR, is the actual sensor of the response. Oxygen could readily oxidize both SoxR forms, but this does not explain the activation of the regulon in anaerobiosis. Gorodetsky *et al.* (2008) have argued that oxidized pyocyanins could be the physiological electron acceptor in *Pseudomonas*, but the identity of the oxidant(s) in enterobacteria is unknown.

Surprisingly, alterations in NADPH levels, including those caused by oxidative stress, were accompanied by inversely correlated changes in total (NADP⁺ + NADPH) contents (Fig. 1), so that they were not translated directly to NADPH:NADP⁺ ratios. A major increase in the total (NADP⁺ + NADPH) pool has been reported for the transition from anaerobic to aerobic lifestyles in *E. coli* (Brumaghim *et al.*, 2003), and in the closely related species *Salmonella enterica* (Grose *et al.*, 2006). NADP⁺ is synthesized from NAD⁺ in a reaction catalysed by NAD⁺ kinase. This enzyme does not belong to the *soxRS* regulon (Blanchard *et al.*, 2007), and its activity did not increase

when *E. coli* cells were exposed to micromolar concentrations of H_2O_2 (Brumaghim *et al.*, 2003). However, Grose *et al.* (2006) showed that NAD^+ kinase is reversibly inhibited *in vivo* by NADPH. Since Brumaghim *et al.* (2003) assayed the activity under standardized *in vitro* conditions (no NADPH present), this effect could have been overlooked. We therefore propose that NADPH consumption by Fd or MV could relieve NAD^+ kinase from feedback inhibition and favour *de novo* synthesis of NADP(H). Conversely, GAPN-expressing cells grown aerobically, with continuous production of NADPH, would keep the enzyme more inhibited than in wild-type bacteria, resulting in lower ($\text{NADP}^+ + \text{NADPH}$) accumulation. Further research will be required to substantiate this contention.

Besides downregulation of the *soxRS* response as demonstrated herein, elevated NADPH levels might also have additional pro-oxidant effects by acting as electron donors for the synthesis of hydroxyl radicals ($\text{OH}^\cdot$) via an Fe^{2+} -dependent Fenton-type mechanism. Both NADPH and NADH can boost this reaction by reducing Fe^{3+} to Fe^{2+} , either directly (Brumaghim *et al.*, 2003) or as substrates for flavin reductase (Woodmansee & Imlay, 2002). Then, reduced pyridine nucleotides could have either protective or damaging effects during oxidative stress episodes, and therefore their cellular concentrations must be tightly regulated to ensure the welfare of the stressed organism.

The effects of oxidants on pyridine nucleotide metabolism have shed some light on the nature of this regulation. On exposure of *E. coli* cells to sublethal H_2O_2 concentrations, two NADPH-producing enzymes were induced: G6PDH and respiration-dependent NADH-NADP⁺ transhydrogenase, together with many scavenging and protective enzymes that oxidize NADPH (Brumaghim *et al.*, 2003). In contrast, most NAD(H)-dependent dehydrogenases and reductases failed to respond to the oxidative challenge (Brumaghim *et al.*, 2003). An even sharper labour division among NADH- and NADPH-linked enzymes occurs in the soil microbe *Pseudomonas fluorescens*, which does not deploy a *soxRS*-type response. On exposure to the superoxide propagator menadione, several NADPH-dependent enzymes were induced, most conspicuously G6PDH and isocitrate dehydrogenase. In this prokaryote, NAD^+ -reducing dehydrogenases were downregulated (Singh *et al.*, 2007).

Determinations of NADH and NADPH levels in aerobically grown enterobacteria have yielded conflicting results. Using [^{14}C]nicotinamide labelling of *E. coli* cultures, Andersen & von Meyenburg (1977) measured equivalent amounts of both reduced pyridine nucleotides. Similar results were obtained by Fuhrer & Sauer (2009) and by Grose *et al.* (2006) in *S. enterica*, whereas Brumaghim *et al.* (2003) observed much higher levels of NADH. Such discrepancies could be due to the different growth media and conditions employed. However, a common feature in most reports is that when cells are exposed to oxidative challenges or adverse environments, the NADH pool tends to be depleted faster than the NADPH pool (see, for

instance, Brumaghim *et al.*, 2003). Preservation of NADPH is advantageous to the cell because the nucleotide can be used as hydride donor by the repair and scavenging enzymes recruited during the stress response. Woodmansee & Imlay (2002) and Brumaghim *et al.* (2003) have argued that this shift also favours tolerance because NADPH is 16-fold less reactive than NADH in the reduction of transition metals and a worse substrate for flavin reductase, therefore reducing the amount of $\text{OH}^\cdot$ radicals formed via Fenton chemistry.

We propose another advantage of the NADH-to-NADPH shift, at least in *E. coli*, which results from the possibility that by increasing NADPH levels it could self-regulate the *soxRS* response. G6PDH is rapidly induced at the onset of the *soxRS* response (Giró *et al.*, 2006), leading to early accumulation of NADPH that could be used by scavenging and repair enzymes such as FPR, flavodoxin and OxyR-dependent reductases and peroxidases, all NADPH consumers. As NADPH contents increase, however, higher activity of SoxR reductases will favour reduction and inactivation of the SoxR sensor, switching off the entire response. It is therefore likely that NADPH accumulation occurs early during *soxRS* induction and proceeds against the activities of FPR and other reductases until the regulon is self-restrained by negative feedback regulation. Through this modulation, facultative aerobes such as *E. coli* could control the dynamic interconversion of their pyridine nucleotides to obtain the maximal benefit from their chemical properties for defence and protection.

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