Oxidative stress

Gisela Storz* and James A Imlay†

Much has been learnt about oxidative stress from studies of *Escherichia coli*. Key regulators of the adaptive responses in this organism are the SoxRS and OxyR transcription factors, which induce the expression of antioxidant activities in response to $O_2^{\bullet-}$ and H_2O_2 stress, respectively. Recently, a variety of biochemical assays together with the characterization of strains carrying mutations affecting the antioxidant activities and the regulators have given general insights into the sources of oxidative stress, the damage caused by oxidative stress, defenses against the oxidative stress, and the mechanisms by which the stress is perceived. These studies have also shown that the oxidative stress responses are intimately coupled to other regulatory networks in the cell.

Addresses
*Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA; e-mail: storz@helix.nih.gov
†Department of Microbiology, University of Illinois, Urbana, Illinois 61801, USA; e-mail: jimlay@uiuc.edu

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Introduction

Oxidative stress is caused by exposure to reactive oxygen intermediates, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO•), which can damage proteins, nucleic acids, and cell membranes. Increasing evidence suggests that the cumulative damage caused by reactive oxygen species contributes to numerous diseases (reviewed in [1]). Recent studies also suggest that the effects of these oxidants are integrally linked to the damage caused by hypochlorous acid (HOCl), and the reactive nitrogen intermediates nitric oxide (NO•), peroxynitrite (HOONO), and nitrosothiols (RSNO). To counter oxidative stress, cells constitutively express enzymes that detoxify the reactive oxygen species and repair the damage caused by them. In addition, bacterial, yeast and mammalian cells all have adaptive responses to elevated levels of oxidative stress, indicating that these cells sense increased levels of reactive oxygen species and transduce the signal into increased expression of defense activities. *Escherichia coli* has been an ideal model for elucidating both the constitutive and adaptive responses to the oxidative stress encountered during routine aerobic growth. In this review, we focus on oxidative stress in *E. coli* but contrast what is known about this organism with the responses in other prokaryotes.

Sources of oxidative stress

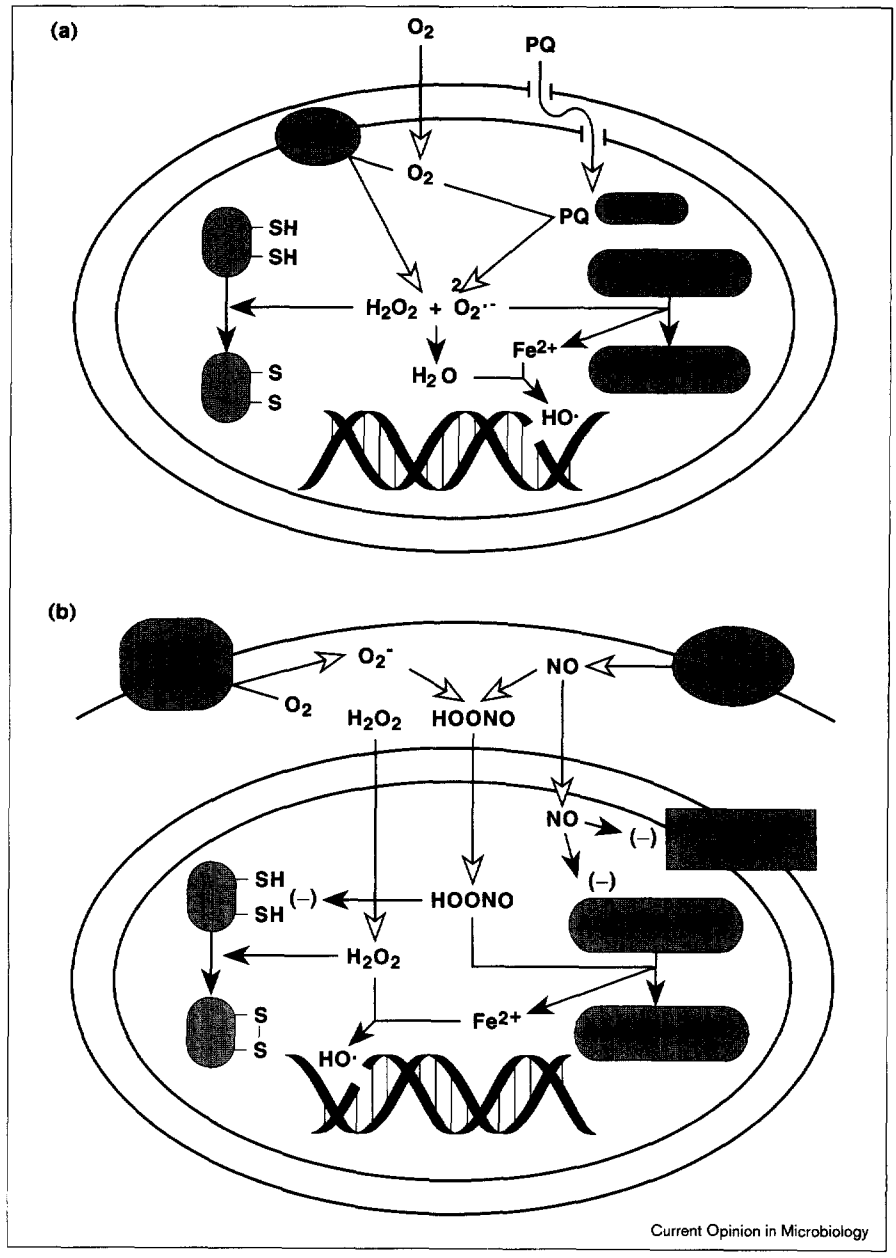
Oxidative stress is an unavoidable by-product of the aerobic lifestyle, because $O_2^{\bullet-}$ and H_2O_2 are formed whenever molecular oxygen chemically oxidizes electron carriers (Figure 1a). Reduced flavoproteins in particular have been implicated in this process in *E. coli*. In exponentially growing *E. coli*, both $O_2^{\bullet-}$ and H_2O_2 are generated by the auto-oxidation of components of the respiratory chain [2,3]. The flavin of NADH dehydrogenase II is the primary site of electron transfer to oxygen in the aerobic respiratory chain; contrary to expectation, little or no $O_2^{\bullet-}$ or H_2O_2 are formed by quinone oxidation or during oxygen reduction at the cytochrome oxidases [4]. Fumarate reductase, a terminal oxidase that is induced during anaerobic growth, reacts very rapidly with oxygen and may confer particular oxidative stress when cells transit from anaerobic to aerobic environments [5]. The expression of both NADH dehydrogenase II and fumarate reductase is regulated in *E. coli* and the enzymes are not present in all bacteria; thus the amount of endogenous oxidative stress will be affected by growth circumstance and will vary from organism to organism. Aerobic *E. coli* synthesizes enough superoxide dismutase to maintain the steady-state $O_2^{\bullet-}$ from these endogenous sources at about 10^{-10} M. This $O_2^{\bullet-}$ concentration is tolerable, about half what is necessary to diminish the activities of vulnerable enzymes and inhibit cell growth [6]. Steady-state H_2O_2 concentrations are higher (10^{-7} to 10^{-6} M) [2] but are still beneath the H_2O_2 toxicity threshold (10^{-5} M) (JA Imlay, unpublished data). Thus the defenses maintained by *E. coli* are calibrated to just avoid toxicity from endogenous oxidants.

These defenses are inadequate, however, if the rates of intracellular $O_2^{\bullet-}$ and H_2O_2 formation are accelerated. Plants, other microorganisms and animals exploit this vulnerability to attack bacterial competitors. For example, plants and some microorganisms secrete redox-cycling antibiotics that diffuse into the competing bacteria, chemically oxidize redox enzymes and transfer the electrons to molecular oxygen. The ecological significance of this tactic is indicated by the presence of adaptive mechanisms in *E. coli* to exclude these antibiotics (reviewed in [7]). In animals, phagocytes employ NADPH oxidase, nitric oxide synthase, and myeloperoxidase to bombard captured bacteria with $O_2^{\bullet-}$, NO•, HOCl, and their chemical by-products, H_2O_2, HO•, HOONO, and RSNO (Figure 1b). Although these enzymes contribute to the killing of bacteria *in vivo*, it is not yet clear which products are directly responsible for toxicity. Unlike the other chemicals, $O_2^{\bullet-}$ (pKa = 4.8) cannot cross membranes at neutral pH [8]. It may, however, conceivably do so in the acidic pH of the phagolysosome. Each of these reactive oxygen and nitrogen species is bacteriostatic or bacteriocidal *in vitro*, but their impacts *in vivo* will depend upon their ultimate concentrations, currently unknown, inside the captured bacterium.

Mechanisms of oxidative cell damage

$O_2^{\bullet-}$ and H_2O_2 have different chemical reactivities and generate distinct types of damage inside cells (Figure 1a).

Figure 1



Mechanisms of oxidative cell damage. **(a)** The process of cell damage by endogenous oxidants. Molecular oxygen passively diffuses into cells and is converted to $O_2^{\bullet-}$ and H_2O_2 by the direct oxidation of flavoproteins, including NADH dehydrogenase II (NdhII). Redox-cycling drugs, including paraquat (PQ), accelerate the formation of these oxygen species by catalyzing the transfer of electrons from redox enzymes such as sulfite reductase (SiRase) to oxygen. $O_2^{\bullet-}$ oxidatively destroys iron–sulfur clusters (here, from aconitase, Acn). The released iron can react with H_2O_2 to form hydroxyl radical HO•, which directly damages DNA. H_2O_2 can also directly oxidize protein cysteinyl residues. **(b)** Plausible contribution of reactive oxygen and nitrogen species to cell damage during phagocytosis. $O_2^{\bullet-}$ and NO• are generated by NADPH oxidase and NO• synthase, respectively, on the phagolysosomal membrane. NO• diffuses passively into the cell where it inhibits the function of aconitase (Acn) and cytochrome oxidase. HOONO is formed by the extracellular reaction of $O_2^{\bullet-}$ and NO•. It diffuses into the cell and attacks cysteinyl residues and iron–sulfur clusters. H_2O_2, also formed outside the bacterial cell, oxidizes cysteine residues and, in conjunction with free iron, the DNA. These toxic reactions have been demonstrated with bacterial cells in culture but have not been tested in phagocytosed bacteria. The white arrowheads indicate the sources of the reactive oxygen and nitrogen species and the black arrowheads denote the damage that is caused.

Mutants of *E. coli* that lack cytosolic superoxide dismutase cannot grow in air without amino acid supplements, cannot catabolize non-fermentable carbon sources, and exhibit high rates of spontaneous mutagenesis ([9]; reviewed in [10]). Most of these phenotypes have been traced to a single type of injury: the oxidative inactivation of a family of dehydratases. These enzymes utilize exposed iron–sulfur clusters [4Fe–4S] to bind and dehydrate substrates; dehydratase oxidation by $O_2^{\bullet-}$ provokes cluster disintegration and a loss of enzyme activity [11]. The auxotrophy of superoxide dismutase mutants for branched-chain amino acids and their inability to catabolize non-fermentable carbon sources reflect the inactivation of dihydroxyacid dehydratase and of aconitase and fumarase, respectively (reviewed in [10]). A by-product of iron–sulfur cluster damage is that copious iron is released into the cytosol, where it catalyzes the oxidation of DNA in conjunction with H_2O_2 (see below) [12,13].

Although H_2O_2 can inhibit cell growth, the causal lesions have not been clearly demonstrated. H_2O_2, however, efficiently oxidizes enzyme thiols, and thus is likely to inactivate enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, that rely upon active-site cysteine residues for catalytic function. H_2O_2 also reacts with adventitious Fe^{2+} to form HO•, a powerful oxidant that reacts at diffusion-limited rates with most biomolecules. Because iron can localize along the phosphodiester backbone of nucleic

Table 1

Antioxidant activities in *Escherichia coli*.

Gene	Activity	Regulators*
sodA	Manganese superoxide dismutase	SoxRS†, ArcAB, FNR, Fur, IHF
fumC	Fumarase C	SoxRS†, ArcAB, σ^s
acnA	Aconitase A	SoxRS†, ArcAB, FNR, Fur, σ^s
zwf	Glucose-6-phosphate dehydrogenase	SoxRS†
fur	Ferric uptake repressor	SoxRS†, OxyR
micF	RNA regulator of *ompF*	SoxRS†, OmpR, LRP
acrAB	Multidrug efflux pump	SoxRS†
tolC	Outer membrane protein	SoxRS†
fpr	Ferredoxin reductase	SoxRS†
fldA	Flavodoxin	SoxRS†
nfo	Endonuclease IV	SoxRS†
sodB	Iron superoxide dismutase	
sodC	Copper–zinc super oxide dismutase	σ^s, FNR
katG	Hydroperoxidase I	OxyR, σ^s
ahpCF	Alkyl hydroperoxide reductase	OxyR
gorA	Glutathione reductase	OxyR, σ^s
grxA	Glutaredoxin 1	OxyR
dps	Non-specific DNA binding protein	OxyR, σ^s, IHF
oxyS	Regulatory RNA	OxyR
katE	Hydroperoxidase II	σ^s
xthA	Exonuclease III	σ^s
polA	DNA polymerase I	
recA	RecA	RecA, LexA
msrA	Methionine sulfoxide reductase	
hslO	Molecular chaperone	
mutM (*fpg*)	8-hydroxyguanine endonuclease	FNR [70]
hmp	Flavohemoglobin	MetR

*The list of regulators is undoubtably incomplete. It is likely that other regulators will be discovered as the antioxidant genes are further studied. †The expression of almost all SoxRS-regulated genes is also modulated by MarA and Rob.

acids, DNA is a particular target of HO•, and most of the cell death that occurs upon H_2O_2 exposure is probably due to DNA damage [14]. A wide variety of DNA lesions are formed (reviewed in [1]). Since some of the base damage can result in miscoding, lesions formed by endogenous oxidants may be a significant or even preponderant source of 'spontaneous' mutagenesis in aerobically growing cells.

The reactive nitrogen intermediates that are released by phagocytes can potentially toxify bacteria by several routes (Figure 1b). NO• blocks bacterial respiration *in vitro* by binding the heme and/or copper sites of cytochrome oxidases [15,16], and it inactivates aconitase through iron–sulfur chemistry that is currently undefined [17]. HOONO, formed by reaction between NO• and $O_2•^-$, is an oxidant that, like $O_2•^-$, rapidly oxidizes dehydratase clusters [18–20] and, like H_2O_2, oxidizes protein thiols [21]. HOONO can also spontaneously isomerize to an activated form, denoted HOONO*, that is powerful enough to oxidize DNA directly [22,23]. This isomerization, however, is slow compared to the other possible fates of HOONO and may not occur in physiological environments. RSNOs can be produced chemically by reaction of HOONO with thiols alone [24] and by reaction of NO• with thiols in the presence of oxygen or metals [25]. RSNOs in turn can stimulate thiol oxidation. Which of these injuries limits the growth and survival of exposed bacteria is currently unsettled.

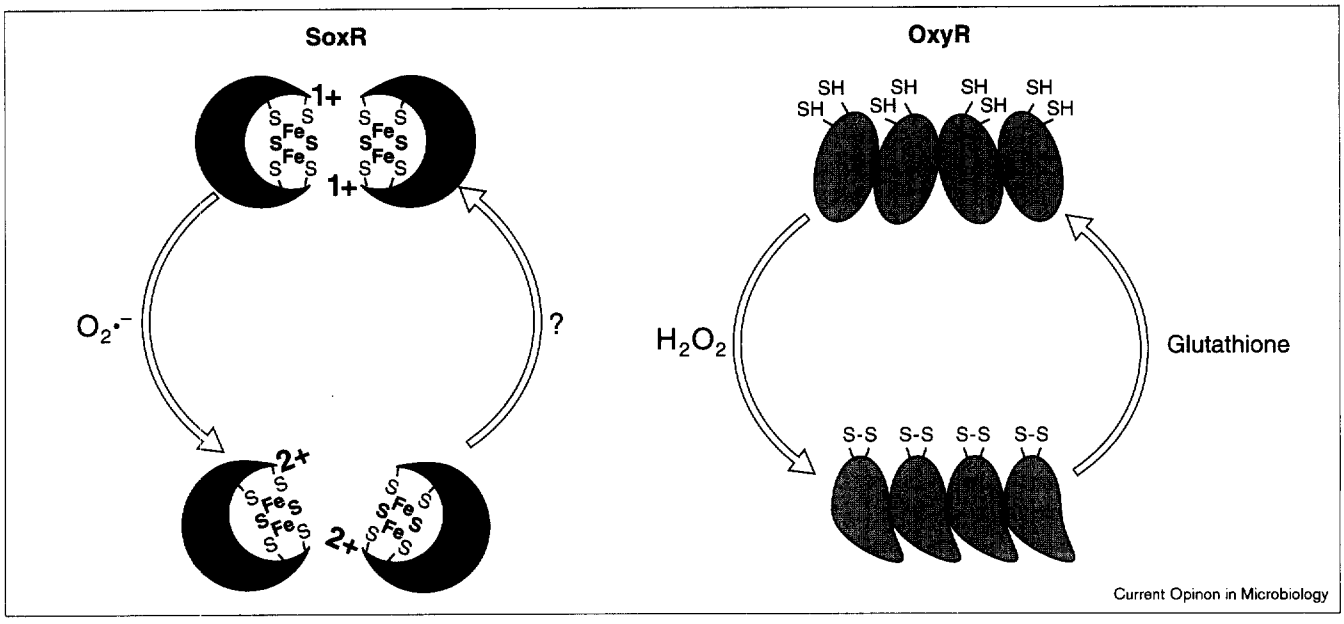
Defenses against oxidative stress

To protect against the damage caused by oxidative stress, cells possess a number of antioxidant enzymes and repair activities, most of which are expressed at low levels during normal growth (Table 1). In response to elevated concentrations of $O_2•^-$ and H_2O_2, the expression of many antioxidant proteins is induced. Among the $O_2•^-$-inducible activities that are regulated by SoxRS transcription factors are manganese superoxide dismutase (encoded by *sodA*), the DNA repair enzyme endonuclease IV (*nfo*), and $O_2•^-$-resistant isozymes of fumarase (*fumC*) and aconitase (*acnA*) (reviewed in [26,27]; SM Varghese, JA Imlay, unpublished data). SoxRS activation also leads to increased levels of glucose-6-phosphate dehydrogenase (*zwf*), which increases the reducing power of the cell, and elevated levels of the Fur repressor (*fur*), which may decrease iron uptake and therefore diminish the formation of •OH ([26,27]; M Zheng, B Doan, TD Schneider, G Storz, unpublished data). The *tolC*-encoded outer membrane protein, the *acrAB*-encoded drug efflux pump, and the MicF regulatory RNA, which represses the expression of the outer membrane porin, all are likely to exclude redox-active compounds that lead to increased $O_2•^-$ levels [7,26,27,28]. The roles of the SoxRS-induced flavodoxin A (*fldA*) and ferrodoxin/flavodoxin- $NADP^+$ reductase (*fpr*) are unknown, although they might function to maintain the reduced state of Fe–S clusters ([26,27]; M Zheng, B Doan, TD Schneider, G Storz, unpublished data). The protective roles of other members of the SoxRS regulon, such as GTP cyclohydrolase II (*ribA*) and two proteins of unknown function (*inaA* and *pqi5*), are not established [29–31].

Two enzymes that clearly protect against $O_2•^-$ damage, but not regulated by SoxRS, are the cytosolic iron superoxide dismutase (*sodB*) and the periplasmic copper–zinc superoxide dismutase (*sodC*) [10,32]. It is also conceivable that an *E. coli* homolog of NifS, which was identified as a protein that provides sulfur for Fe–S cluster assembly in *Azotobacter vinelandii*, might protect against $O_2•^-$ stress, but neither the expression nor the physiological role of this gene has been studied extensively in *E. coli* [33].

The expression of many of the H_2O_2-inducible activities is regulated by the OxyR transcription factor, including hydroperoxidase I (catalase, *katG*), the two subunits of an alkyl hydroperoxide reductase (*ahpCF*), glutaredoxin 1 (*grxA*), glutathione reductase (*gorA*), and the Fur repressor (*fur*) ([26,27,34,35•]; M Zheng, B Doan, TD Schneider, G Storz, unpublished data). The phenotypes of mutations in the OxyR-regulated genes *dps* and *oxyS* indicate that the

Figure 2



Mechanisms of SoxR and OxyR activation and deactivation. Interestingly, the redox-active center of SoxR, which is directly or indirectly activated by $O_2^{\bullet-}$, an oxidant that damages iron–sulfur clusters, is an iron–sulfur cluster, whereas the redox-active center of OxyR, which is directly activated by H_2O_2, an oxidant that oxidizes protein cysteinyl residues, is a redox-sensitive disulfide bond. SoxR and OxyR also respond to NO• and RSNO, respectively.

nonspecific DNA-binding protein Dps and the OxyS regulatory RNA protect against mutagenesis [36,37]. Interestingly, the crystal structure of Dps revealed that the protein is a ferritin homolog, suggesting that Dps may protect against DNA damage by sequestering iron [38•]. The roles of three other recently identified OxyR targets, *hemF* (encoding a coproporphyrinogen III oxidase), *rcsC* (a regulator of capsular polysaccharide synthesis genes) and *f497* (encoding a protein with homology to arylsulfatase enzymes), are not understood [39]. OxyR negatively regulates the expression of the outer membrane protein antigen 43 (*agn43*) gene, which also does not have an obvious role in the oxidative stress response [40].

Activities that protect against H_2O_2, but are not under OxyR control include hydroperoxidase II (*katE*) and the DNA repair enzymes exonuclease III (*xthA*), DNA polymerase I (*polA*) and RecA [14,26,27]. Peptide methionine sulfoxide reductase (*msrA*) and the molecular chaperone Hsp33 (*hslO*), whose activity is redox-regulated, also protect against oxidative stress but do not appear to be regulated by OxyR [41,42•].

In addition to protecting against $O_2^{\bullet-}$- and H_2O_2-induced damage, the SoxRS regulon provides resistance to many different drugs as well as organic solvents and reactive nitrogen species [7,43,44], and OxyR-regulated activities have been found to confer resistance to HOCl, organic solvents, and reactive nitrogen species [45–47,48•]. For example, the OxyR-regulated AhpC protein protects cells against reactive nitrogen intermediates, an AhpC activity that is independent of the AhpF subunit required for protection against peroxides [48•]. An important direction for future studies will be to elucidate all of the functions of the members of the SoxRS and OxyR regulons. Additional defense activities and regulators are also likely to be identified. For example, bacterial flavohemoglobin (*hmp*) has been identified as an NO•-detoxifying enzyme [49•–51•]. *hmp* expression is induced by nitrosylated compounds, and recent studies suggest that this regulation is dependent on the MetR transcription factor [52].

Regulation of the defenses against oxidative stress

Significant progress has been made toward elucidating the mechanisms by which the activities of the SoxR and OxyR transcription factors respond to $O_2^{\bullet-}$ and H_2O_2 stress, respectively (Figure 2). Regulation of the *soxRS* regulon occurs by a two-stage process: the SoxR protein is first converted to an active form that enhances *soxS* transcription, and the increased level of SoxS in turn activate expression of the regulon (reviewed in [26,27]). The constitutively expressed SoxR protein contains two [2Fe–2S] centers per dimer. The oxidation of the reduced $[2Fe–2S]^{1+}$ form of SoxR to a $[2Fe–2S]^{2+}$ form appears to be the mechanism of SoxR activation [53–55,56•,57•,58] (Figure 2). Evidence for this mechanism comes from experiments in which the Fe–SoxR protein reduced with dithionite was found to regain transcriptional activity upon auto-oxidation [54]. Electron paramagnetic resonance spectroscopy of whole cells also has shown that over-produced wild-type SoxR protein is oxidized within two minutes after cells are treatment with

$O_2^{\bullet-}$-generating compounds, and constitutively active mutant SoxR proteins are predominantly in the oxidized form even in the absence of stress [56•,57•,58]. The nature of the signaling molecule that activates SoxR is still under debate. The Fe–SoxR protein might be oxidized directly by $O_2^{\bullet-}$ or indirectly by other molecules in the cell. Gort and Imlay [6] observed very little induction of *soxS* expression in a superoxide dismutase deficient strain suggesting that SoxR senses some signal other than $O_2^{\bullet-}$, however, others (P Gaudu, D Touati, personal communication; SI Liochev, I Fridovich, personal communication) do detect moderate *soxS* induction in superoxide dismutase deficient strains. Liochev *et al.* [59] suggest that SoxR activity may be modulated by alterations in NADPH or reduced flavodoxin or ferredoxin levels, but Gaudu and Weiss [54] were unable to observe measurable reduction of SoxR by these compounds *in vitro*. Any model for SoxR activation needs to take into account that the protein is also activated by NO• [44]. Although the mechanism of SoxR reduction/deactivation has not been elucidated, monothiols have recently been found to promote the disassembly, whereas dithiols promote the assembly of the [2Fe–2S] clusters [60]. Clearly, the nature of the $O_2^{\bullet-}$-stress signaling molecule and the mechanisms of SoxR activation and deactivation are important directions for future study.

The tetrameric OxyR protein exists in two forms, reduced and oxidized, but only the oxidized form activates transcription (Figure 2). Thus direct oxidation of OxyR by H_2O_2 is the mechanism whereby the cells sense oxidative stress and induce the OxyR regulon. OxyR is also activated by reaction with RSNO [47], but H_2O_2 is a more effective inducer [35•]. Mutational studies suggested that OxyR oxidation involved one, possibly two, cysteine residues [61]. Mass spectrometric analysis and thiol-disulfide titrations showed that an intramolecular disulfide bond is formed between residues Cys199 and Cys208 upon OxyR oxidation [35•]. The formation of the disulfide bond has been proposed to proceed via a sulfenic acid intermediate, but the nature of the intermediate and the basis of the unusual reactivities of Cys199 and Cys208 to H_2O_2 need to be investigated further. *In vivo* and *in vitro* studies showed OxyR is reduced and deactivated by enzymatic reduction with glutaredoxin 1 [35•]. Thus, because the expression of glutaredoxin 1 and glutathione reductase is induced by OxyR, the response is autoregulated.

Overlap between oxidative stress response and other regulatory networks

Several other transcriptional regulators in addition to SoxR and OxyR modulate the expression of antioxidant genes, illustrating the extensive connectivity between the SoxRS/OxyR regulons and other regulatory networks. The *rpoS*-encoded σ^S subunit of RNA polymerase is important for the expression of a large group of genes that are induced when cells encounter a number of different stresses including starvation, osmotic stress, and acid stress as well as upon entry into stationary phase (reviewed in [62]). Starved and stationary phase cells are intrinsically resistant to a variety of stress conditions including high levels of H_2O_2, and σ^S has been shown to regulate the expression of several antioxidant genes including *katE*, *xthA*, and *sodC* [32,62]. The SoxRS-regulated *pqi5* gene and the OxyR-regulated *katG*, *gorA* and *dps* genes are also part of the σ^S regulon [62].

The anaerobic regulators FNR and ArcAB also modulate the of expression of the SoxRS-regulated *sodA*, *acnA* and *fumC* genes, and FNR controls *sodC* [32,63–65]. In addition, two SoxS homologs, MarA and Rob, regulate the expression of almost all genes in the SoxRS regulon [7]. Finally, the expression of one transcription factor, Fur, is modulated by both SoxRS and OxyR (M Zheng, B Doan, TD Schneider, G Storz, unpublished data). It is likely that many more connections among the *E. coli* the regulatory networks will be discovered.

Broad distribution of regulators

In this review we have focused on the *E. coli* responses to oxidative stress. The characterization of antioxidant activities and the corresponding regulators in other prokaryotes has pointed to similarities and also some interesting differences between *E. coli* and other bacteria. Several conclusions can be made from a survey of these studies. First, oxidative stress responses are not exclusive to aerobes. Superoxide dismutase and catalase activities have been observed in anaerobes, and the anaerobic bacterium *Bacteroides fragilis* clearly has an adaptive response to H_2O_2 [66]. The recent isolation of a peroxide-resistant *B. fragilis* mutant that overexpresses catalase, alkyl hydroperoxide reductase subunit C and a Dps homolog should allow the identification of the regulator of this response [66].

Second, although OxyR homologs have been characterized in a number of bacteria, the arrangement of genes surrounding *oxyR* is different in each organism: in *E. coli* and *Salmonella typhimiurim*, the *oxyS* gene is upstream of and on the opposite strand to *oxyR*; in *Mycobacterium*, *ahpC* is upstream of and on the opposite strand to *oxyR*; and in *Xanthomonas*, *ahpC*, *ahpF* and *oxyR* are adjacent and in the same orientation with one transcript encoding *ahpC* and a second transcript encoding *ahpF* and *oxyR* [37,67,68]. Thus while the regulators and the antioxidant genes have been conserved, the corresponding genes have been shuffled.

Third, the presence of reactive oxygen species may be sensed by regulators that are distinct from SoxR and OxyR. A good example is the PerR repressor of *Bacillus subtilis* [69•]. PerR is a Fur-like, metal-binding protein that represses the expression of catalase, an alkyl hydroperoxide reductase, and Dps-like protein. It is postulated that PerR activity might be regulated by metal-catalyzed oxidation of the protein or by a change of the oxidation of a bound metal ion [69•]. The additional characterization of regulators such as PerR may bring to light novel mechanisms of redox-sensing.

Conclusions

Further characterization of the *E. coli* responses to oxidative stress will undoubtedly reveal many more important

and possibly unexpected clues about cellular responses to oxidative stress. Another important direction for future work will be to examine oxidative stress responses of other prokaryotes especially under special circumstances, such as the oxygenation of obligate anaerobes and the oxidative assault upon bacteria in the phagolysosome.

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