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Diagnosing oxidative stress in bacteria: not as easy as you might think

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Abstract

Microorganisms are vulnerable to elevated levels of intracellular reactive oxygen species (ROS). This situation has led to proposals that many natural stresses might be toxic specifically because they accelerate endogenous ROS formation. Such a mechanism has been convincingly demonstrated for redox-cycling compounds. However, the evidence is much weaker for most other stressors. The hypothesis that clinical antibiotics generate lethal ROS stress has attracted much attention, and the author discusses some aspects of evidence that support or oppose this idea. Importantly, even if all cellular electron flow were somehow diverted to ROS formation, the resultant doses of H_2O_2 and O_2^- would more likely be bacteriostatic than bacteriocidal unless key defense mechanisms were simultaneously blocked.

Introduction

Life evolved in an anoxic world, and so contemporary organisms have inherited biochemical features that are substantially incompatible with the presence of oxygen. In fact, both calculations and experiments indicate that microbes have acquired just enough defensive measures to avoid overt poisoning by endogenous reactive oxygen species (ROS) [1]. Any elevation in the intracellular levels of these oxidants—notably, superoxide (O_2^-) and hydrogen peroxide (H_2O_2)—produces enough enzyme damage that growth stalls and enough DNA damage that mutagenesis accelerates.

Since life is poised on this knife's edge, investigators often wonder whether various stressors might exert their toxic effects by amplifying the natural rate of ROS production (Table 1). A variety of experimental approaches have been used to test these ideas. The results do not always provide a consensus, and the purpose of this review is to explore why seemingly straightforward analyses can produce data that are ambiguous or contradictory.

A particular example of this problem is the debate over clinical antibiotics. Work from many groups, spearheaded by the Collins and Walker labs [2–6], has provided evidence that

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aminoglycosides, β-lactams, and fluoroquinolones owe some of their lethal effects to the generation of ROS. Other groups are unconvinced and cite contrary data [7–12]. The author is mainly in the latter camp and will describe the nature of key elements of the evidence. Due to length restrictions, this discussion is not comprehensive. Readers are encouraged to read a recent review to learn an opposing viewpoint [13].

Escherichia coli is the model system in which the details of oxidative stress are best understood. ROS are continuously formed in oxic *E. coli* through the adventitious autoxidation of its redox enzymes; their accumulation is held in check by the superoxide dismutases that degrade O_2^- and the peroxidases and catalases that degrade H_2O_2 (Fig. 1). Mutants that lack either set of enzymes suffer damage to specific iron enzymes and cannot grow under conditions in which their activities are necessary [14–19]. DNA damage also increases due to reactions between H_2O_2 and the intracellular pool of labile iron [20–22]:

$$Fe^{2+} + H_2O_2 \rightarrow [FeO^{2+}] + H^+ + OH^- \rightarrow Fe^{3+} + OH^- + HO.$$

The hydroxyl radicals thus formed react avidly with virtually all biomolecules. Their reactions with DNA produce some lesions that are misread by DNA polymerase and others that block its progress. If enough of the latter lesions are generated, replication never recovers, and the cell dies. This scenario has been proposed to explain some part of the lethal action of antibiotics [13].

Do redox-cycling drugs generate oxidative stress? Theory and evidence

It is useful to consider the example of redox-cycling compounds—paraquat, menadione, phenazines, and others—in thinking about how oxidative stress might arise and be detected inside bacteria. These agents include natural compounds that are released by plants and bacteria in an effort to suppress the growth of competitors. The chemical foundation for their action is well-understood. These compounds are facile acceptors and donors of single electrons, and in vitro it can be shown that they non-specifically oxidize many redox enzymes by abstracting electrons from their exposed flavin and metal centers [23]. In fact, the rate of electron transfer from enzymes to these agents can match or exceed the rate of transfer to physiological acceptors. The semiquinone species thus formed then rapidly pass the electrons to molecular oxygen. Superoxide is thereby produced—and, through its dismutation, H_2O_2 is generated as well [24–26].

These redox-cycling compounds can penetrate into cells like *E. coli*, and they easily impair the growth of aerobic but not anoxic bacteria [24,26], which suggests that they are poisonous precisely because they drive ROS production. Intracellular ROS formation has been confirmed in several ways. The exposed cells continue to consume oxygen even when cyanide is added to block cytochrome oxidases; this “cyanide-resistant respiration” is a marker of illicit oxygen reduction by the drugs [25,26]. Strains that lack catalases and peroxidases naturally release H_2O_2 into growth medium—and this rate is greatly augmented when redox-cycling compounds are added [7]. The OxyR regulatory protein, which directly senses H_2O_2, becomes activated [27]. ROS-sensitive dehydratases lose activity [15,28]. Mutants that lack SOD are hypersensitive [14,28]. Mutation rates rise [20,29]. In fact, the cell features a regulon (SoxRS) whose purpose is to directly sense these redox agents; it

responds by inducing the synthesis of superoxide dismutase and of endonuclease IV, an enzyme that repairs oxidative DNA damage [30,31].

Thus a battery of measurements provide a consensus: these compounds amplify ROS formation and thereby debilitate the cell. These techniques are therefore the obvious choice to test other suspected sources of ROS stress. Interestingly, the amount of DNA damage that redox-active compounds cause is usually not sufficient to kill cells—a point that will be germane to the antibiotic issue.

Do clinical antibiotics impose oxidative stress? Evidence from ROS measurements

The oxidative-stress hypothesis of antibiotic action is representative of ROS-overproduction theories; it is an especially useful example because its proponents have employed a range of creative approaches to test the idea. The notion that standard antibiotics might create ROS stress arose initially from microarray analyses [2]. The data showed that the SoxRS, Fur, and IscR regulons were partially activated when *E. coli* was exposed to barely toxic doses of norfloxacin. At the time of this work it was not recognized that SoxR directly senses drugs rather than O_2^- [32], and so these data were interpreted to mean that both ROS and labile iron pools might somehow be perturbed. It seemed logical that the sequelae might include an increase in DNA lesions, potentially contributing to the loss of viability.

To test this idea, the investigators used redox-sensing dyes as a means of appraising oxidative stress inside living cells. They checked whether chemical antioxidants and cell-permeable iron chelators would slow cell death. They also tested whether cells would be protected by the overproduction of ROS scavenging enzymes and DNA repair enzymes. All of these approaches generated data that appeared to support the ROS hypothesis [3].

However, most of the conventional markers that had successfully detected oxidative stress in the case of redox-cycling compounds did not give any such indication in the case of clinical antibiotics. Both the original microarray experiment and subsequent analyses by RT-PCR and gene fusions did not show significant activation of the OxyR regulon [2,3,7]. This outcome was surprising, because OxyR is the natural mechanism by which the cell senses threatening levels of H_2O_2. The rate of H_2O_2 effusion from catalase/peroxidase mutants was unchanged [7]. ROS-sensitive dehydratases did not lose activity, and labile iron pools, which typically swell from oxidative damage to iron enzymes, did not grow [7]. Mutants that lack SOD or catalase/peroxidase were not particularly sensitive [7,10]. The same was true of *oxyR*, *recA*, and *polA* mutants, all of which are rapidly killed by authentic H_2O_2 [7,10]. (One exception: the DNA repair mutants were hypersensitive to norfloxacin, in accord with its direct action upon topoisomerase.)

Thus the genetic and biochemical markers that worked so well for redox-cycling compounds all came up negative for classical antibiotics. How can we account for the discrepancy?

Do dyes work?

Reduced fluorescein and rhodamine dyes can be oxidized by hydroxyl radicals in vitro (Fig. 2) [33,34]. When these dyes are loaded into cells, the subsequent application of exogenous H_2O_2 enhances fluorescence, confirming that these indicators can detect rapid Fenton chemistry in vivo as well. Because the oxidation process involves a radical intermediate, there is an opportunity for unrelated chemical events to affect the product yield, and experts caution that perturbations of CO_2, glutathione, and SOD levels, for example, can be misinterpreted as a change in the rate of hydroxyl formation [33,34].

However, three other issues potentially complicate the interpretation of bacterial experiments. First, the dyes can be oxidized not only by hydroxyl radicals but also by high-valence metal centers, including the compound I form of heme enzymes and even cytochrome c [7,33–36]. Thus metabolic effects that alter the redox states of such enzymes can, at least in principle, generate a read-out that could be misconstrued as evidence of ROS stress. Ideally the involvement of Fenton chemistry in dye oxidation would be confirmed by checking whether the fluorescence yield depends upon the titer of catalase/peroxidase.

Second, disruption of the membrane barrier can affect the amount of the dye that penetrates into cells. This is true not only because an intact lipid bilayer sterically excludes such compounds but also because pmf-dependent pumps may actively excrete them [33]. Aminoglycoside antibiotics in particular disrupt the cytoplasmic membrane and increase the infusion of small molecules, including dyes [37]. The proper control is to normalize the fluorescent signal to the amount of dye inside the cells. Such ratiometry is common to the probe methods that measure other metabolites, and it is standard for the use of peroxidase-based and boronate-dependent H_2O_2 sensors [38,39]. However, it has not yet become the usual practice in fluorescent-dye experiments.

Finally, bacterial cell dimensions can change during protracted stress. If cells become large, their fluorescence rises proportionately, even if there is no difference in internal fluorophore concentration. Two labs independently demonstrated that this artifact undermined initial cell-sorting experiments that appeared to show high ROS after antibiotic treatment, as both ampicillin and norfloxacin triggered cell filamentation [11,12]. Measurements of side- and back-scatter, or of the natural fluorescence of dye-free cells, reveal the problem. The better approach is to normalize the fluorescence of the total cell population to its biomass; alternatively, if cell sorting is desired, the data must be normalized to side-scatter or to simultaneous measurements of a GFP-tagged housekeeping protein.

Dye-based ROS methods have found a broad clientele because the manipulations are straightforward. However, interpretation is ambiguous unless proper controls are performed. Interested readers are advised to review expert literature [33,34].

Can chemical “antioxidants” suppress oxidative damage?

Glutathione, ascorbate, and vitamin E have frequently been added to bacterial cultures in an effort to suppress ROS phenotypes. These compounds are natural antioxidants in mammalian systems. Glutathione is the reductive substrate for glutathione peroxidase, the

primary scavenger of H_2O_2, and ascorbate plays the same role for ascorbate peroxidase. However, neither enzyme is present in *E. coli*, and by themselves these chemicals do not directly degrade H_2O_2 or O_2^- at a useful rate [40]. Vitamin E suppresses lipid peroxidation in eukaryotic organisms by reducing lipid radicals. However, the absence of polyunsaturated fatty acids means that lipid peroxidation is not likely to happen in most bacteria [41,42]. Thus there is no rationale for the use of any of these compounds as diagnostic antioxidants in bacteria.

Cell-penetrating iron chelators like dipyridyl and desferrioxamine quickly block Fenton chemistry and thereby prevent ROS from damaging targets such as DNA [43]. However, when bacteria are incubated with these compounds for extended periods of time, the resultant iron starvation triggers adjustments in metabolism with wide-ranging effects on growth and energy charge. Dipyridyl was found to protect *E. coli* from aminoglycoside antibiotics not because it prevented hydroxyl radical production but because the diminished membrane potential inhibited antibiotic import [10].

Can enzyme overproduction identify the damage mechanism?

Bacteria respond to threats by inducing defensive enzymes, and the identities of those enzymes can point to the damage mechanism. For example, the protective effect of superoxide dismutase induction helped to confirm that O_2^- is central to the mechanism by which redox-cycling compounds poison cells [44]. A complementary approach is to artificially overexpress defensive enzymes to see whether they suppress injuries.

A potential problem with this strategy is that the excessive synthesis of any enzyme can debilitate cells and hinder growth. Slow-growing cells are generally less vulnerable to killing mechanisms, so misinterpretations can ensue. A clever control is the parallel overproduction of inactive mutant forms of the defensive protein [6]. For example, overproduction of superoxide dismutase, NADH peroxidase, catalase, MutT, and MutS all diminished the rates at which classical antibiotics killed *E. coli*, and mutant forms of these enzymes were less protective. However, in those experiments a powerful plasmid/promoter system was used, and even the mutant proteins provided notable protection. Constitutive induction of defensive proteins from their native promoters, through use of a modified form of OxyR, did not provide any protection [10].

The reciprocal experiment is to diminish the levels of putatively protective enzymes. In the antibiotic example, mutants lacking the ROS scavenging enzymes—superoxide dismutase, NADH peroxidase, and catalase—were no more sensitive than wild-type cells [7,10].

Is it plausible that E. coli could make enough H_2O_2 to kill itself?

Some oxidative stress models posit that stressors accelerate the rate of H_2O_2 production simply by elevating flux through the respiratory chain [3,6]. This seems unlikely, since the chain appears not to be the primary site of basal ROS formation [45]. A derivative model is that enzymes within the chain are partially disabled by stress, thereby enhancing their tendency to directly leak electrons to oxygen. Could this generate enough ROS to kill cells?

It is hard to kill cells with achievable levels of ROS. The awareness that HO· is potentially lethal came from ionizing radiation experiments. In retrospect, this was a misleading example, since ionizing radiation creates spurs of radicals that generate multiple lesions close together on DNA. Double-stranded breaks result and are often fatal. Fenton chemistry appears not to work in that way: lesions are dispersed, and DNA-repair processes are generally successful.

Experiments confirm that oxidative stress is much more likely to inhibit growth than to kill cells. Based on direct measurements of H_2O_2 production and catalase/peroxidase levels, the steady-state level of H_2O_2 inside unstressed aerobic *E. coli* has been projected to be ~ 50 nM [1]. Studies have used catalase/peroxidase mutants to conveniently establish higher steady-state levels of H_2O_2 within cells. As little as 0.5 μM intracellular H_2O_2 can block growth in a defined medium by inactivating mononuclear iron enzymes [46], and higher doses inactivate iron-sulfur dehydratases such as those involved in amino-acid synthesis (Fig. 3A). However, even 8 μM intracellular H_2O_2 does not produce enough DNA damage to cause death, as evidenced by the resumption of growth when amino acids are provided (Fig. 3) [17]. Since the steady-state level of H_2O_2 in wild-type cells is proportionate to the rate of its formation, a > 150-fold increase in endogenous H_2O_2 production—which would be needed to establish an 8 μM intracellular level—would apparently still be non-lethal. Under standard conditions about 0.3% of the oxygen consumption leads to H_2O_2 formation, and so a 150-fold increase would represent an unrealistic 45% of oxygen consumption. Thus for any stressor to impose lethal ROS damage, it must do more than simply accelerate H_2O_2 formation. Indeed, in our studies redox-cycling compounds like paraquat have been bacteriostatic but not bacteriocidal.

Micromolar H_2O_2 has been observed to kill *E. coli* cultures in the lab only when the cells were aerated in iron-rich LB medium *and* carried mutations in *oxyR, dps, recA, polA*, or *xthA* [47] (Fig. 3B). The OxyR protein induces Dps, a ferritin-like protein that sequesters iron. The latter three genes encode key enzymes in recombinational (*recA*) or excision (*polA, xthA*) DNA-repair pathways. These observations still allow the possibility that exogenous stressors might impose ROS- driven death, but to do so those stressors would need to disable defenses as well as elevate ROS formation. There is no evidence yet that antibiotics are able to do this. For example, iron levels did not rise during antibiotic treatment [7].

Conclusions

When workers propose that oxidative damage is responsible for the lethal effects of an exogenous stress, they face two challenges. The first is to unambiguously demonstrate that a stress truly does raise the level of intracellular ROS. Unfortunately, the experimental techniques that are most direct are not always easy or widely applicable. The second challenge is that only special, contrived circumstances have been found in which micromolar H_2O_2 can be lethal. Mechanisms that simultaneously disable key oxidative defenses would be required. This may surprise people: even the doses of H_2O_2 that accumulate in phagosomes (< 10 μM [48–51]) may not be directly cidal.

The quest for new and better antibiotic therapies is an urgent one, and the examination of cell physiology during antibiotic action may provide helpful ideas. However, the author hesitates to accept the notion that oxidative stress is an important contributor to antibiotic lethality. A simple model that antibiotics generate lethal doses of oxidative damage would require that a very large fraction of oxygen consumption be diverted towards H_2O_2 formation; this prediction was not supported by a battery of assays that have easily detected other ROS stresses. This outcome conflicts with the data from experiments with ROS probes. Potential problems with those probes have been repeatedly highlighted in expert reviews, and cell-sorting analysis may introduce additional complications. Other approaches that have led workers to the oxidative-stress hypothesis—protection by chemical antioxidants and by protein overproduction—also have complications that must be considered. At this point one hopes that new measures of oxidative stress might be developed that can resolve the mixed results obtained thus far.

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Highlights

1. Workers have hypothesized that many different stresses accelerate ROS formation.
2. Common measures of ROS formation can be subverted by artifacts.
3. ROS are typically bacteriostatic rather than bacteriocidal.
4. The evidence that antibiotics impose oxidative stress is mixed.

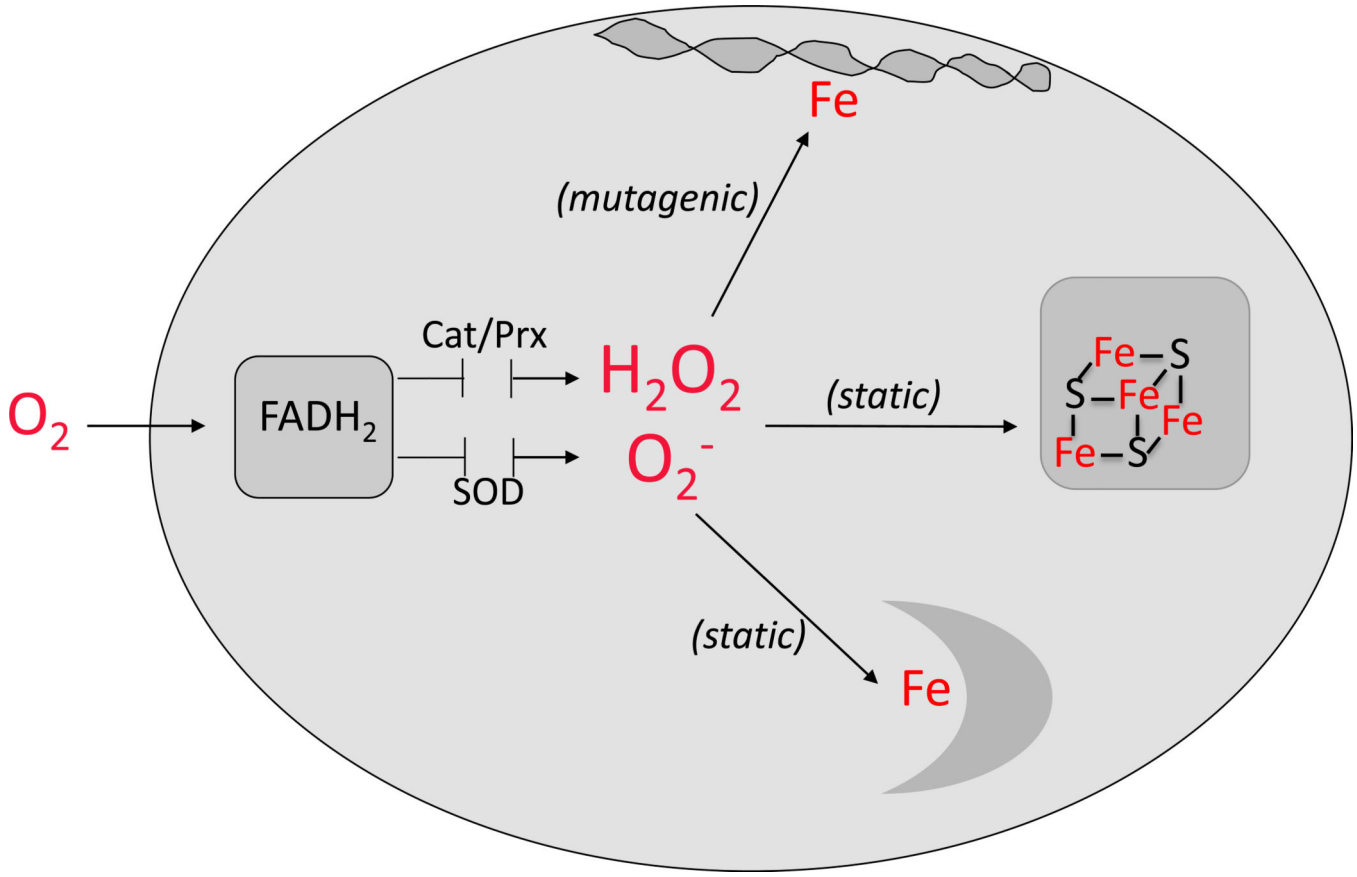

Figure 1. Targets of ROS in *E. coli*

Autoxidation of redox enzymes generates O_2^- and H_2O_2 that are kept at low concentrations by scavenging enzymes such as catalases (Cat), peroxidases (Prx), and superoxide dismutases (SOD). Any boost in H_2O_2 concentration accelerates reactions with the pool of free iron; DNA damage and mutagenesis ensue. Excess O_2^- and H_2O_2 inactivate Fe/S-dependent dehydratases and mononuclear Fe enzymes, thereby disabling pathways and blocking growth.

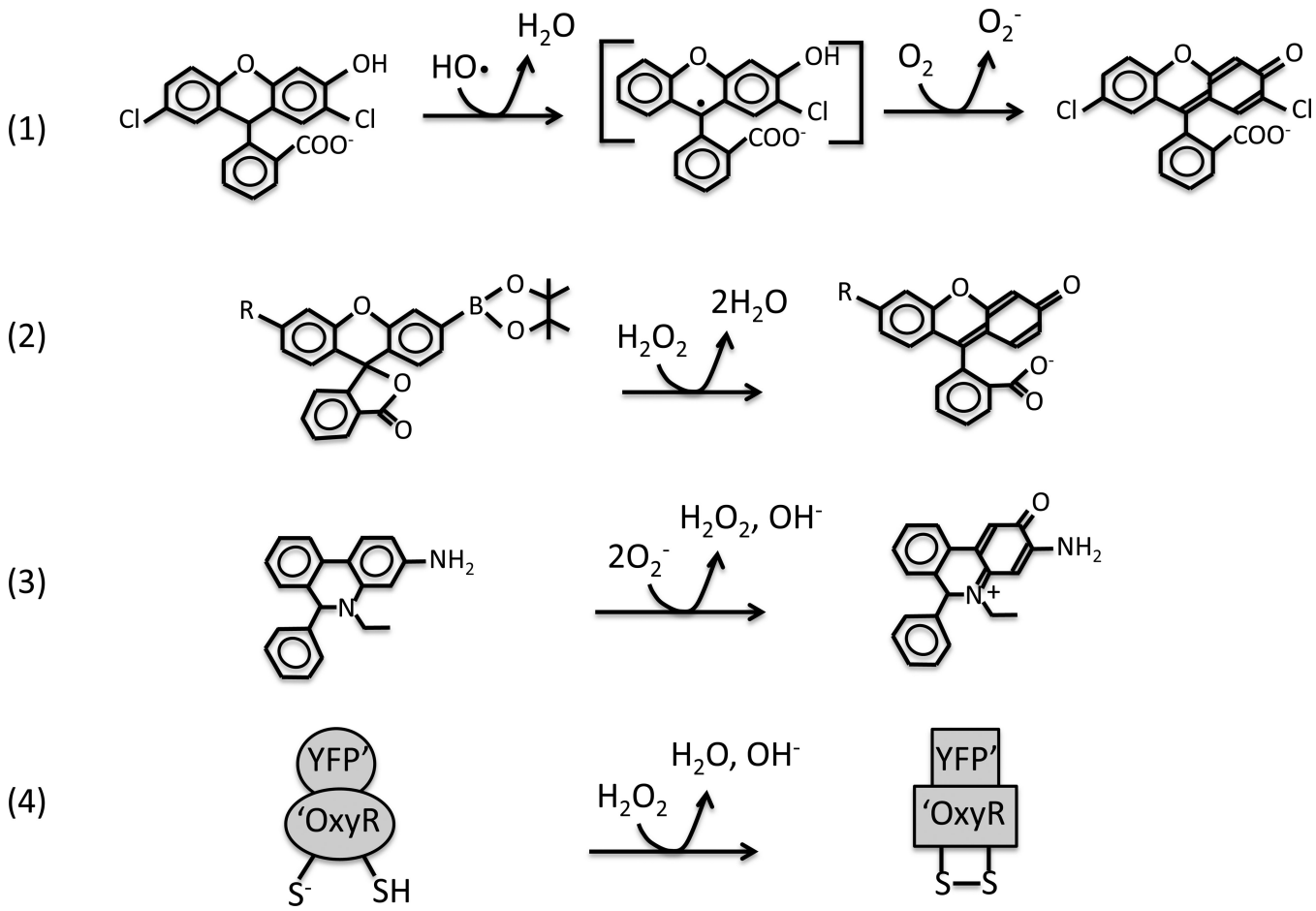

Figure 2. Intracellular fluorescent probes of ROS

(A) Reduced fluorescein derivatives (here, dihydrodichlorofluorescein) can be oxidized in a two-step process initiated by HO·. Carbonate radicals, high-valence metals, and other strong univalent oxidants can also initiate the reaction [33]. (B) Boronate-caged probes developed by the Chang group [39] react directly with H_2O_2. These probes have detected an influx of 100 μM intracellular H_2O_2 into eukaryotic cells. Ratiometric methods can correct for loading discrepancies. (C) Hydroethidine can be oxidized by O_2^- to 2-hydroxyethidium. Oxidized metal centers can oxidize the probe to a distinct ethidium derivative that can be distinguished by its fluorescence spectrum [77]. The detection limit in vivo has not been determined. (D) HyPer [38] is an artificial protein in which the H_2O_2-sensing domain of OxyR is fused to the fluorescent domain of YFP. Oxidation shifts the fluorescence spectrum. This probe is reversible in vivo, like OxyR itself, and has detected an infusion of 5–25 μM H_2O_2 into *E. coli*.

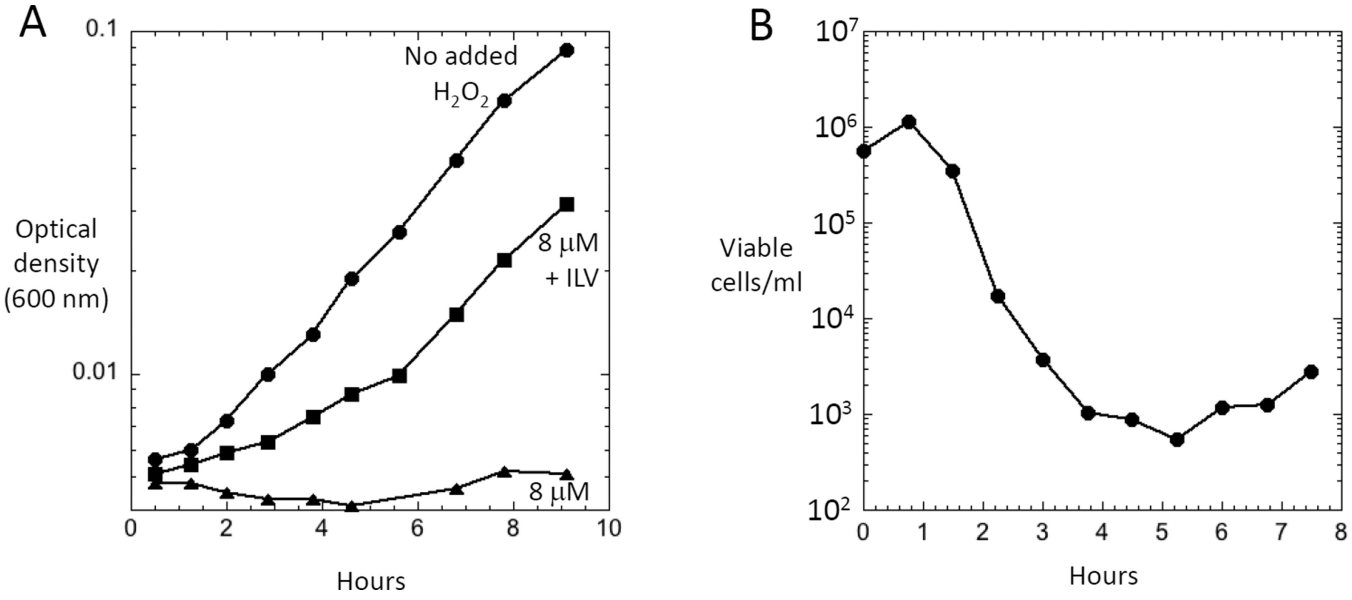

Figure 3. Impact of elevated H_2O_2 upon cell fitness

(A) Catalase/peroxidase mutants that are suffused with 8 μM H_2O_2 exhibit an auxotrophy for branched-chain amino acids due to dehydratase inactivation. The figure indicates growth curves in defined glucose medium to which exogenous H_2O_2 was added. If branched-chain amino acids (ILV) are supplied, cells can grow. (All media also contained aromatic amino acids to counteract inhibition of that biosynthetic pathway. Data are from Jang and Imlay [17].) (B) Substantial cell death from H_2O_2 stress occurs only if catalase/peroxidase mutants additionally carry a Δ*dps* mutation that blocks the sequestration of intracellular iron by this mini-ferritin. Anoxic cells growing in liquid medium were aerated at time zero, and at intervals the number of surviving cells was determined by plating on anoxic plates. This phenotype was evident in LB medium containing ~ 10 μM Fe and ~ 5 μM H_2O_2. Data are from Park *et al.* [47].

Table 1

The list is incomplete. Where possible, literature was cited that employs *E. coli* as a model system. The involvement of ROS in toxicity is generally accepted in some cases but is less settled in others.

Some proposed triggers of ROS stress	Sample reference[1]
Ionizing radiation	[52]
Hyperoxia	[53]
Quinones	[25]
Viologens	[24]
Phenazines	[26]
Hydroxyurea	[54]
Tannins	[55]
Clinical antibiotics	[3]
Amoebae	[56]
Plant wound response	[57]
Phagocytic oxidative burst	[58]
Lactic acid bacteria	[59]
Heat	[60]
Hydrostatic pressure	[61]
Salinity	[62]
Near-UV radiation	[63]
Photosynthesis	[64]
Alcohol	[65]
Iron overloading	[21]
Silver	[66]
Copper	[67]
Chromate	[68]
Cobalt	[69]
Tellurite	[70]
Nanoparticles	[71]
Senescence	[72]
Phosphate starvation	[73]
Peptidoglycan recognition proteins	[74]
Cytotoxic proteases	[75]
Addiction cassettes	[76]