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Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*

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Abstract

Very little manganese is imported into *Escherichia coli* under routine growth conditions: the import system is weakly expressed, the manganese content is low, and a manganese-dependent enzyme is not correctly metallated. Mutants that lack MntH, the importer, grow at wild-type rates, indicating that manganese plays no critical role. However, MntH supports the growth of iron-deficient cells, suggesting that manganese can substitute for iron in activating at least some metalloenzymes. MntH is also strongly induced when cells are stressed by hydrogen peroxide. This adaptation is essential, as *E. coli* cannot tolerate peroxide stress if *mntH* is deleted. Other workers have observed that manganese improves the ability of a variety of microbes to tolerate oxidative stress, and the prevailing hypothesis is that manganese does so by chemically scavenging hydrogen peroxide and/or superoxide. We found that manganese does not protect peroxide-stressed cells by scavenging peroxide. Instead, the beneficial effects of manganese correlate with its ability to metallate mononuclear enzymes. Because iron-loaded enzymes are vulnerable to the Fenton reaction, the substitution of manganese may prevent protein damage. Accordingly, during H₂O₂ stress, mutants that cannot import manganese and/or are unable to sequester iron suffer high rates of protein oxidation.

Introduction

When organisms dwell in aerobic habitats, molecular oxygen enters cells and adventitiously oxidizes intracellular redox enzymes, thereby producing a continuous stream of intracellular superoxide and hydrogen peroxide. These reactive oxygen species (ROS) are kept at tolerable steady-state concentrations by the actions of superoxide dismutases and reductases and of peroxidases and catalases (Imlay, 2008).

However, microorganisms are perfused with higher levels of H₂O₂ when they enter environments in which H₂O₂ has accumulated as a consequence of the chemical oxidation of reduced metals, sulfur species, or organic molecules. Microbes are also exposed to high H₂O₂ concentrations when other organisms use it as a weapon to poison their competitors. A variety of lactic acid bacteria excrete H₂O₂ at rates that lead to millimolar concentrations in culture media, while some plants and other bacteria export redox-cycling compounds that trigger ROS formation when they are ingested by cohabitant microbes. Finally, the phagosomal NADPH oxidase sprays captive microbes with very high doses of H₂O₂.

In these circumstances the basal scavenging systems are likely to be inadequate, and intracellular H₂O₂ can accumulate to toxic (micromolar) levels (Seaver & Imlay, 2001b). This H₂O₂ reacts with unincorporated ferrous iron to generate hydroxyl radicals, which then rapidly oxidize nearby biomolecules; when this reaction involves iron that is bound to DNA,

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mutagenic or lethal lesions can result (Levin *et al.*, 1982, Henle *et al.*, 1999, Imlay *et al.*, 1988, Park *et al.*, 2005). H₂O₂ also inactivates a family of dehydratases by oxidizing their solvent-exposed iron-sulfur clusters (Jang & Imlay, 2007, Flint *et al.*, 1993). As a consequence, the catabolic and biosynthetic pathways that rely upon these enzymes become non-functional.

To cope with this threat, microbes have evolved inducible defensive regulons that are controlled by H₂O₂-sensing regulatory proteins—OxyR (Carmel-Harel & Storz, 2000) and PerR (Lee & Helmann, 2006) in bacteria, and Yap-1 (Paget & Buttner, 2003) in yeast. In *E. coli*, intracellular H₂O₂ stress activates the OxyR transcription factor, which then directly stimulates synthesis of approximately two dozen proteins (Zheng *et al.*, 2001). The protective roles of about half of these are understood. The AhpCF peroxidase and the KatG catalase directly limit H₂O₂ accumulation (Seaver & Imlay, 2001a). Dps is a ferritin-like protein that sequesters unincorporated iron and thereby minimizes Fenton chemistry and, consequently, DNA damage (Grant *et al.*, 1998, Ilari *et al.*, 2002, Park *et al.*, 2005). The elevated expression of Fur protein, a repressor of iron-import genes, further limits the amount of intracellular iron (Varghese *et al.*, 2007, Zheng *et al.*, 1999). The *suf* genes encode an iron-sulfur-cluster assembly complex that replaces the customary Isc machinery (Takahashi & Tokumoto, 2002, Lee *et al.*, 2004), which evidently is also poisoned by H₂O₂ (Jang and Imlay, in preparation).

The roles of the other OxyR-controlled genes are not yet known. One of these is *mntH*, which encodes the only dedicated manganese importer in *E. coli* (Kehres *et al.*, 2000b). Studies in a variety of other microbes have indicated that manganese can help protect cells against oxidative stress. Manganese supplementation allowed *Streptococcus pneumoniae* (Tseng *et al.*, 2002) and *Neisseria gonorrhoeae* (Seib *et al.*, 2004) to better tolerate millimolar levels of H₂O₂ that might otherwise have been lethal. *Deinococcus radiodurans*, which is exceptionally resistant to ionizing radiation, lost a large component of that resistance when it was cultured in medium that limited the availability of manganese (Daly *et al.*, 2004).

The basis of these protective effects is not clear. Manganese is an essential cofactor of manganese-containing superoxide dismutase, but this enzyme is not co-regulated by OxyR, as one might expect it to be if the intended effect of *mntH* induction were to charge this enzyme. Instead, many workers have proposed that manganese might mitigate oxidative stress by chemically scavenging superoxide and/or hydrogen peroxide. Manganese complexes exhibit these activities in vitro (Horsburgh *et al.*, 2002b, Gray & Carmichael, 1992, Berlett *et al.*, 1990), although the rate constants depend upon their coordinating ligands and, in the best cases, are still far lower than those of enzymic manganese-dependent superoxide dismutases and Mn-catalases.

The goal of this study was to inspect the role of MntH generally and during oxidative stress in particular. To do so we exploited strains of *E. coli* that lack catalases and peroxidases (Hpx⁻). When cultured aerobically, these strains accumulate up to 1 μM of intracellular (and extracellular) H₂O₂ (Seaver & Imlay, 2001b), a constant stress that moderately exceeds the dose (ca. 0.1 μM) that activates expression of the OxyR regulon (Aslund *et al.*, 1999). We show here that in this situation, a failure to induce MntH results in a profound growth defect. However, our analysis suggests that the requirement for manganese import stems from a need to cofactor enzymes rather than to act as a chemical scavenger of ROS.

Results

Manganese import into unstressed cells is minimal and expendable

E. coli has two cytoplasmic superoxide dismutases (SODs): an iron-dependent isozyme (FeSOD, encoded by *sodB*) (Yost & Fridovich, 1973) and a manganese-dependent isozyme (MnSOD, encoded by *sodA*) (Keele *et al.*, 1970). Activity-gel experiments by Pugh *et al.* indicated that MnSOD activity was elevated by an order of magnitude when manganese was added to standard defined growth medium (Pugh *et al.*, 1984). This result could not be explained by the known mechanisms of *sodA* regulation, and it led workers to suggest that under routine growth conditions cells may not contain enough manganese to activate the apoenzyme. Using *sodB* mutants that express only the MnSOD isozyme, we confirmed that MnSOD activity rose from 2 U/mg to 40 U/mg when cells were grown in manganese-supplemented medium. A *sodA'*-*lacZ*⁺ transcriptional fusion was not induced by this treatment, as β -galactosidase activity was 3.2 \pm 0.06 U/mg under routine conditions and 2.9 \pm 0.04 U/mg after manganese supplementation.

To test the enzyme-activation idea directly, cell extracts were prepared from *sodB* mutants and assayed immediately in order to quantify MnSOD activity. The extracts were then treated with denaturants, demetallated with the chelators EDTA and 8-hydroxyquinoline-5-sulfonic acid, and then renatured in the presence of manganese in order to fully activate the MnSOD proteins. The denaturation/renaturation protocol boosted the initial extract activity by at least twenty-fold, indicating that < 5% of the enzyme molecules recovered from cells contained manganese in their active sites (Fig. 1A, bars 1 and 2). In contrast, 60% of the MnSOD was already active when it was recovered from manganese-supplemented cells (Fig. 1A, bars 3 and 4). Similar results were obtained from AB1157 and W3110 derivatives of *E. coli* K-12 (data not shown).

MnSOD activity in unsupplemented cells was boosted about six-fold when *mntH* was provided (behind its own promoter) on a multicopy plasmid (Fig. 1A, bars 5 and 6). Thus the failure to fully metallate MnSOD *in vivo* derives from inadequate expression of the manganese importer. Several labs have shown that when MnSOD is recovered from iron-rich cells, a significant fraction contains iron and is therefore inactive (Beyer & Fridovich, 1991, Whittaker & Whittaker, 1997). The preceding experiments were conducted at low cell densities in iron-sufficient medium, raising the possibility that inadequate manganese uptake allowed iron to outcompete manganese for binding to the apo-protein. However, follow-up experiments failed to distinguish whether the inactive MnSOD proteins contained iron or lacked metals entirely.

Reciprocal experiments with *sodA* mutants were performed to test the metallation status of FeSOD. Less than a 10% increase in activity was achieved by demetallation and remetallation with ferrous iron, indicating that the as isolated FeSOD enzymes were fully loaded with iron. Collectively, these data indicate that when wild-type cells were cultured in standard media, they contained sufficient iron to fully metallate newly synthesized apo-FeSOD, while intracellular manganese was too scarce to effectively metallate apo-MnSOD. Indeed, ICP measurements indicated that the total intracellular manganese concentration was only 15 μ M (Fig. 2), compared to nearly 1 mM for iron ((Outten & O'Halloran, 2001); data not shown).

These results also suggest that *E. coli* can thrive even when it does not import enough manganese to metallate manganese-dependent enzymes. To more rigorously test this conclusion, we deleted the gene that encodes the only specific manganese transporter in *E. coli*, *MntH*. The manganese content of these mutants was minimal (Fig. 2), and MnSOD

activity was virtually absent (Fig. 1, bars 7 and 8). Nevertheless, growth was as robust as that of wild-type cells (Fig. 1B).

Cells require manganese import during H₂O₂ stress

These results raise the question: Under what circumstances is manganese import important for cell fitness? The regulatory proteins that control MntH synthesis may provide clues. One of these proteins, Fur, directly senses iron levels in *E. coli*. When iron is abundant, Fe²⁺-metallated Fur protein binds the promoter region of *mntH* and represses its transcription (Kehres et al., 2000b, Patzer & Hantke, 2001). However, in low-iron conditions Fur is predominately in the apo-form, cannot bind DNA, and no longer inhibits *mntH* transcription. Thus the cell activates manganese import when iron levels are low. Such regulation implies that manganese can compensate for iron deficiency. Indeed, Grass *et al.* have shown that manganese entry through *mntH* can stimulate the growth of iron-import mutants (Grass *et al.*, 2005). We replicated these results (data not shown).

A plausible mechanism for this compensation is by the insertion of manganese into enzymes that normally employ iron as a cofactor. Manganese is unlikely to substitute for iron in iron-sulfur clusters or hemes, since the large disparity between its reduction potential and that of iron would interfere with the redox activities of these cofactors. Instead, it is possible that manganese takes the place of mononuclear iron in enzymes that employ divalent metals to bind substrates. In vitro a variety of metals, including manganese, typically suffice to activate these enzymes. The ability of iron to activate such enzymes has not been well explored. However, recent studies show that enzymes such as isocitrate dehydrogenase (Murakami *et al.*, 2006) and transketolase (Sobota and Imlay, unpublished), which are commonly activated by manganese in vitro, are also fully functional if they are charged with ferrous iron. This raises the possibility that iron may be the usual cofactor for some metal-activated enzymes in vivo. If so, then during periods of iron scarcity the induction of MntH may enable manganese to take its place. This substitution might also spare enough iron to charge those enzymes that require heme and iron-sulfur cofactors.

Another transcription factor, OxyR, induces expression of *mntH* during periods of H₂O₂ stress (Kehres *et al.*, 2000a, Kehres *et al.*, 2002b). The roles of OxyR-controlled genes have been studied in catalase/peroxidase (Hpx⁻) mutants. These strains cannot degrade hydrogen peroxide; therefore, when they are cultured in aerobic media, the adventitious oxidation of intracellular redox enzymes leads to the accumulation of up to 1 μM intracellular H₂O₂ (Seaver & Imlay, 2001b). The OxyR stress response is activated by as little as 0.1 μM H₂O₂ (Aslund et al., 1999), and we confirmed that an *mntH*'-lacZ+ fusion was strongly induced in the Hpx⁻ mutant when it was transferred from anaerobic to aerobic media (Fig. 3). In contrast, very little expression occurred in a wild-type background, consistent with the low level of intracellular manganese and the absence of a null-mutant phenotype.

A deletion mutation of *mntH* was generated in anaerobic Hpx⁻ mutants, without any apparent effect on anaerobic growth (data not shown). However, when the Hpx⁻ Δ*mntH* mutants were diluted into aerobic defined medium, they soon stopped growing (Fig. 4a). This phenotype was reproduced in other *E. coli* K-12 backgrounds and in various types of defined media (data not shown). A plasmid carrying *mntH* restored normal growth, as did either the *katG* or *ahpCF* wild-type alleles. The addition of micromolar concentrations of manganese to the medium also allowed growth—while 100 μM iron, zinc, and copper did not (data not shown)—confirming that the pertinent role of MntH is to import manganese.

This result also indicates that there must be a secondary, low-affinity route by which manganese can enter the cell (Fig. 1A, bars 7-10). That route is unidentified but required 50 μM manganese for full growth stimulation, suggesting that its native substrate may be a

cation other than manganese. In contrast, MntH imported sufficient manganese from the standard defined medium, which contained only $0.14 \pm 0.01 \mu\text{M}$ manganese. Interestingly, the $\text{Hpx}^- \Delta\text{mntH}$ mutants were able to grow in unsupplemented Luria-Bertani (LB) medium, apparently because they accumulated substantial intracellular manganese (Fig. 2). Luria-Bertani (LB) medium contains more manganese ($0.25 \pm 0.01 \mu\text{M}$ manganese) than does defined medium, and unlike the defined medium it lacks phosphate and citrate, metal chelators that can restrict manganese availability. In the Hpx^- mutants the impact of the OxyR-dependent induction of *mntH* was to elevate the intracellular manganese content to $150 \mu\text{M}$, an order of magnitude above that of wild-type cells (Fig. 2). As a consequence, the efficiency of MnSOD activation was much higher (Fig. 4B).

We deleted the OxyR binding site upstream of *mntH*. This mutation did not diminish basal expression, but it eliminated the OxyR-driven induction of the gene during H_2O_2 stress (data not shown). The Hpx^- mutants containing this non-inducible allele exhibited a growth behavior intermediate between that of the $\text{Hpx}^- \Delta\text{mntH}$ strain and its *mntH*⁺ parent (Fig. 4A). Thus basal expression of *mntH* is inadequate for H_2O_2 -stressed cells, presumably because they require supranormal levels of manganese. Indeed, even the Hpx^- (*mntH*⁺) strain grew best when additional manganese was added to the medium (data not shown).

Manganese import is not required to scavenge reactive oxygen species

Previous reports speculated that manganese might protect oxidatively stressed cells because it has the chemical ability to scavenge superoxide and H_2O_2 (Horsburgh et al., 2002b, Gray & Carmichael, 1992, Berlett et al., 1990). The Hpx^- strain suffers specifically from H_2O_2 stress, and so we directly measured the rates at which $\text{Hpx}^- \text{mntH}^+$ and $\text{Hpx}^- \Delta\text{mntH}$ cells degraded H_2O_2 (Fig. 5A). Contrary to the scavenging hypothesis, the MntH^+ cells did not degrade H_2O_2 any more rapidly than did their ΔmntH counterparts. Further, manganese supplements did not provide any activity. More generally, $\text{Hpx}^- \text{mntH}^+$ cells released as much H_2O_2 as did $\text{Hpx}^- \Delta\text{mntH}$ mutants (Fig. 5B). Since the rate of efflux is directly proportional to internal H_2O_2 concentrations (Seaver & Imlay, 2001b), this result indicates that imported manganese did not diminish intracellular H_2O_2 levels.

Superoxide degrades the iron-sulfur clusters of dehydratases, and the released iron can catalyze DNA damage by H_2O_2 (Flint et al., 1993, Liochev & Fridovich, 1994, Keyer & Imlay, 1996, Kuo et al., 1987). This mechanism provides a route by which superoxide can exacerbate the toxicity of H_2O_2 ; therefore, we examined the possibility that imported manganese was needed to suppress Fenton chemistry by activating MnSOD. However, a $\text{Hpx}^- \Delta\text{sodA}$ mutant grew well in defined medium, indicating both that MnSOD activity was expendable and that imported manganese was critical for some other purpose (Fig. 6A). In a complementary experiment, the *sodA* gene was provided on a multicopy plasmid to boost the SOD activity of $\text{Hpx}^- \Delta\text{mntH}$ cells. Still, the cells were able to grow in aerobic medium only if manganese supplements were provided (Fig. 6B). Indeed, when the cells were washed free of the manganese, growth quickly ceased even though the residual SOD activity substantially exceeded that of a wild-type strain. Most tellingly, the *sodA*-overexpressing plasmid actually inhibited the aerobic growth of an Hpx^- strain that contained the wild-type *mntH* allele—a strain which otherwise would have grown well—presumably because the elevated level of MnSOD protein sequestered the intracellular manganese so that it could not perform its critical (unknown) function. Finally, the addition of both ΔsodA and ΔsodB alleles to the Hpx^- strain did not block its growth in this medium (data not shown). All together, these results indicate that Hpx^- growth depends critically upon intracellular manganese rather than upon superoxide scavenging. We conclude that the key role of manganese in these H_2O_2 -stressed cells is something other than the degradation of H_2O_2 or O_2^- .

Manganese does not protect DNA from oxidative damage

A primary target of H₂O₂ is DNA, through Fenton chemistry catalyzed by DNA-bound iron. We evaluated the possibility that manganese might protect the DNA by displacing iron from its surface. The 150 μM manganese that accumulated inside the Hpx⁻ cells was much less than the DNA nucleotide concentration in growing cells (15-30 mM) (Neidhardt & Umberger, 1996), indicating that manganese could never shield the DNA from all potential iron binding. Nevertheless, some DNA sequences create strong metal-binding sites and might be particular loci for damage (Rai *et al.*, 2001), and in principle manganese might displace iron from these sites.

However, microscopic examination showed that Hpx⁻ *ΔmntH* cells did not filament, and despite their poor growth the number of viable cells did not diminish (data not shown). Filamentation and death are two hallmarks of oxidative DNA damage (Park *et al.*, 2005). Further, although ICP analysis confirmed that high-dose manganese supplements raise the manganese content of wild-type cells to about 200 μM, these supplements did not diminish the H₂O₂ sensitivity of DNA-repair-defective mutants: only 1% of *recA* mutants survived a five-minute exposure to 2.5 mM H₂O₂ whether or not the growth medium was supplemented with 100 μM manganese. Finally, manganese supplements did not allow the growth of Hpx⁻ *ΔrecA* cells (data not shown). These latter experiments indicate that excess manganese does not suppress Fenton-mediated DNA damage. We infer that the growth defect of Hpx⁻ *ΔmntH* mutants does not arise from DNA oxidation and that it is more likely to reflect a disruption in cell metabolism.

Manganese is required by the cell to protect enzymes

When Hpx⁻ *ΔmntH* cells were supplemented with manganese, the amount of manganese that was needed to stimulate growth correlated with the amount needed to metallate MnSOD (Fig. 7). Because MnSOD activity per se is not necessary to allow growth, we interpret this correspondance more generally as an indication that H₂O₂-stressed cells need to import enough manganese to ensure the metallation of one or more key enzymes. Aside from MnSOD, there are few or no enzymes in *E. coli* that specifically require manganese for function (Kehres & Maguire, 2003). While many enzymes from *E. coli* can utilize manganese in vitro to satisfy a general requirement for a divalent metal, it is likely that many of these enzymes can use magnesium as an alternative. We knocked out several genes (*nrdEF*, *icd*, *pgmI*, *mntR*, *glpX*, *maeA*) that encode proteins that have been suspected of relying on manganese in vivo, but none of these mutations replicated the *ΔmntH* growth defect in the Hpx⁻ background (data not shown).

It is notable that magnesium binds more weakly than manganese to transketolase A and isocitrate dehydrogenase, perhaps because magnesium is less tolerant of deviations from its ideal coordination geometry (Kehres & Maguire, 2003). If magnesium is a mediocre metal for such enzymes, and manganese is not imported into unstressed cells, then ferrous iron is a plausible candidate to be the usual activating metal. This logic suggests that manganese might serve to metallate such enzymes only during periods of iron starvation or H₂O₂ stress, the two conditions that activate synthesis of MntH.

During H₂O₂ stress the OxyR system also induces Dps, a ferritin-like protein that sequesters loose iron and thereby diminishes the amount of Fenton-based DNA damage (Grant *et al.*, 1998, Ilari *et al.*, 2002, Park *et al.*, 2005). Therefore, one possible rationale for *mntH* induction during H₂O₂ stress would be that the action of Dps effectively starves cells for iron, so that without manganese the divalent-metal-requiring enzymes would be left in an apoprotein form. An alternative hypothesis is that ferrous-iron-loaded enzymes would be targets for Fenton reaction, and that the replacement of iron by manganese allows these

enzymes to avoid oxidative injury. A testable difference between these two ideas is that the first hypothesis suggests that manganese protects the cell when it has too little unincorporated iron, whereas the second implies that manganese protects the cell when it has too much.

EPR-based measurements indicated that Hpx⁻ mutants contain more unincorporated iron than do wild-type cells (Park et al., 2005). The increase is likely due to the oxidative degradation of iron-sulfur clusters (Jang & Imlay, 2007); in any case, this result is more consistent with the second hypothesis rather than the first. Further, we observed that the profound growth defect of Hpx⁻ Δ dps mutants could be suppressed by manganese supplements (Fig. 8A). This result, too, suggests that manganese rescues the cell from iron overload rather than from iron starvation. Finally, iron chelators substantially stimulated the growth of Hpx⁻ Δ mntH cells (Fig. 8B), indicating that the purpose of manganese is to out-compete iron.

These results supported the notion that manganese incorporation might spare enzymes from iron-driven oxidation reactions. Some fraction of Fenton events within proteins generate carbonyl groups on local amino acid residues. These can be detected on Western blots using antibodies against the DNP-derivatized proteins. Proteins extracted from Hpx⁻ Δ mntH mutants exhibited substantially more carbonyls than did proteins from their MntH⁺ parents (Fig. 9). The excess carbonylation was suppressed when manganese was supplemented into their growth medium. Further, the Hpx⁻ Δ dps mutants exhibited extremely high levels of carbonylation, and this damage was also diminished by manganese. These data confirm that excess intracellular iron accelerates protein oxidation, while manganese import suppresses it.

Discussion

Are manganese and iron alternative cofactors for mononuclear-metal enzymes?

Life evolved in an anaerobic, iron-rich world. Photosystem II did not begin to generate molecular oxygen until about 2.8 byr ago, and it is likely that during the subsequent billion years the atmosphere still remained essentially anaerobic, as the nascent oxygen was quickly scavenged by reaction with dissolved ferrous iron in the Earth's seas (Bjerrum & Canfield, 2002). Only later, after the iron had been oxidized and precipitated as ferric minerals, did oxygen accumulate. Thus the fundamental biochemical mechanisms and metabolic pathways that contemporary organisms use were inherited from an environment very unlike our own.

The depletion of bioavailable iron posed a problem for organisms, and they responded by inventing elaborate import systems to satisfy their iron demand. *E. coli* grown in laboratory cultures contains about 1 mM iron (Outten & O'Halloran, 2001), much of it incorporated as a cofactor in enzymes that rely upon heme or iron-sulfur clusters. However, the fact that manganese import is stimulated upon iron depletion (Kehres et al., 2000b, Patzer & Hantke, 2001), and that it can substantially compensate for an insufficient iron supply (Grass et al., 2005), suggests that a substantial fraction of imported iron might be used as a mononuclear cofactor in non-redox enzymes and that manganese can substitute for it. Indeed, although more than sixty *E. coli* enzymes can be activated by manganese in vitro (www.ecocyc.org), the robust growth of *mntH* mutants shows that these enzymes must be able to employ some other metal in vivo. Some of these enzymes are likely to use magnesium, but for others iron is the obvious candidate, since in its ferrous form it shares with manganese(II) a similar size, valence, and coordination geometry. Most manganese-activatable enzymes have not been adequately tested for their ability to use iron, because when such experiments are performed in aerobic buffers, ferrous iron is quickly oxidized to the unusable ferric form. We suspect,

then, that some of these proteins evolved with iron as their routine cofactor, and that metallation by manganese is an alternative that is resorted to only when iron levels are low.

The few mononuclear enzymes that have redox functions may be instructive exceptions. For example, although iron and manganese again compete to bind in the protein coordination environments that are presented by superoxide dismutases, the two metals cannot provide equivalent activity, since they differ in their reduction potentials. To cope with this issue, discrete SOD isozymes are synthesized in *E. coli* and in many other bacteria, one for iron and one for manganese (Yost & Fridovich, 1973, Keele et al., 1970). Under iron-sufficient conditions, the iron-dependent isozyme is synthesized and the manganese-dependent enzyme is substantially repressed (Tardat & Touati, 1993), suggesting that the latter is a back-up enzyme used in circumstances of iron deficiency.

Certain organisms seem to have evolved to not depend on iron for cell chemistry, including *Lactobacillus plantarum* (Archibald, 1986a), *Borrelia burgdorferi* (Posey & Gherardini, 2000) and *Streptococcus suis* (Niven et al., 1999); notably, they have unusually high concentrations of intracellular manganese, which might serve as a cofactor for mononuclear metal enzymes. These bacteria additionally dispense with pathways that depend upon Fe/S- or heme-cofactored enzymes, such as the TCA cycle, and/or employ manganese-based enzymes in their place. For instance, *L. plantarum* uses a heme-less Mn-catalase to scavenge H₂O₂ (Kono & Fridovich, 1983, Barynin et al., 2001).

Manganese is not an efficient scavenger of ROS

The ability of manganese to defend microbes against oxidants has been noted in several contexts: imported manganese protects *Neisseria gonorrhoeae* (Seib et al., 2004, Tseng et al., 2001), *Streptococcus pneumoniae* (Tseng et al., 2002), and *Salmonella typhimurium* (Kehres et al., 2000a) against exogenous H₂O₂, while lactic-acid bacteria, which generate H₂O₂ as a stoichiometric by-product of central metabolism, are rich in intracellular manganese (Archibald, 1986b). Further, *Deinococcus radiodurans* loses its characteristic resistance to ionizing radiation if manganese import is limited (Daly et al., 2004). Collectively these results have prompted suggestions that manganese might serve inside the cell as a chemical scavenger of superoxide (Archibald & Fridovich, 1981, Chang & Kosman, 1989, Inaoka et al., 1999, Tseng et al., 2001, Horsburgh et al., 2002a, Al-Maghrebi et al., 2002, Daly et al., 2004, Sanchez et al., 2005), of hydrogen peroxide (Runyen-Janecky et al., 2006), or of both (Kehres et al., 2000b, Horsburgh et al., 2002b, Kehres & Maguire, 2003, Seib et al., 2004). However, the peroxide-efflux experiments reported here directly show that in *E. coli*, at least, the imported manganese does not effectively degrade H₂O₂. Why not? Stadtman and colleagues showed that the ability of manganese to rapidly disproportionate H₂O₂ requires the formation of a manganese complex with three equivalents of HCO₃⁻ (Berlett et al., 1990). Metabolites are likely to interfere with this activity by coordinating the metal; and, even if they did not, the rate constant of the dismutation reaction is too low to enable physiological concentrations of manganese to effectively degrade H₂O₂. For example, if intracellular bicarbonate were, generously, 28mM and manganese were 150 μM, the half life of H₂O₂ would be 27 min, exclusive of the activities of Ahp and catalase. When the latter are considered, the half-life would be 1.3 msec (calculated from (Seaver & Imlay, 2001b)). In other words, the scavenging activities of these enzymes exceed the chemical activity of this concentration of manganese by six orders of magnitude. This calculation explains why imported manganese did not alter H₂O₂ efflux, and it confirms that the role of MntH induction is not to augment the scavenging activity of Ahp and KatG. It also suggests why *L. plantarum*, which has a high intracellular content of manganese, nevertheless synthesizes a dedicated Mn-catalase.

What about superoxide dismutation? This reaction can be accomplished by manganese that is loosely bound to certain biomolecules, with rate constants that reached 13,200 SOD units/mg Mn(II) in the best case (Archibald & Fridovich, 1982). In a cell containing 150 μM manganese, this ideal complex would provide 108 U/mL of dismutation activity. That amount still pales, however, compared to the 3000 U/mL enzymatic SOD present in unstressed cells (Imlay & Fridovich, 1991) and the 14,000 U/mL in H_2O_2 -stressed *E. coli* (calculated from this study). For both superoxide and H_2O_2 dismutation reactions, the kinetic efficiency depends upon the midpoint potential of the Mn(II)/Mn(III) couple and therefore is strongly affected by the coordination environment. The purpose of SOD and catalase proteins is to control that environment. So it seems unlikely that cells would rely on manganese that is loosely deposited on the surfaces of various biomolecules to serve this catalytic role. Interestingly, while there has been great interest in the use of manganese chelates as therapeutic sources of SOD activity, experiments have indicated that these drugs may not work by scavenging superoxide (Munroe *et al.*, 2007). One alternative is that they serve as cell-permeable carriers of manganese, which then acts to metallate and protect mononuclear metal enzymes.

The OxyR regulon is dedicated to the suppression of Fenton chemistry

The OxyR regulon has been calibrated by evolution to be activated by about 0.1 μM intracellular H_2O_2 , suggesting that this dose is physiologically relevant (Aslund *et al.*, 1999). Because the cell membrane is only semi-permeable to hydrogen peroxide, an extracellular concentration of 1 to 5 μM H_2O_2 is probably what activates this system inside a wild-type (scavenger-proficient) cell (Seaver & Imlay, 2001b); therefore, this is the concentration that one would like to impose in order to study the physiology of oxidative stress. However, it is difficult to establish and maintain this dose in experimental cultures of substantial cell density: wild-type cells rapidly degrade H_2O_2 that is provided in a single bolus (e.g., Fig. 5A), and it is not easy to arrange continuous H_2O_2 production in order to replicate the steady-state H_2O_2 levels that might occur in nature. As an alternative, catalase/peroxidase-deficient (Hpx^-) strains permit workers to impose steady, physiological H_2O_2 stresses for a long enough period of time so that growth defects can become apparent.

To date the debilitating effects of micromolar H_2O_2 stress have all been tracked to Fenton-type reactions, including the oxidation of DNA (Park *et al.*, 2005), the destruction of dehydratase iron-sulfur clusters (Jang & Imlay, 2007), and the de-regulation of iron homeostasis through the inactivation of Fur protein (Varghese *et al.*, 2007). The rate constant of the Fenton reaction ranges from 10^3 - $10^4 \text{ M}^{-1} \text{ s}^{-1}$ for these biological targets. Thus growth defects arise and mutagenesis becomes prominent when intracellular H_2O_2 concentrations reach 1 μM , and it is fitting that the OxyR protein becomes activated when H_2O_2 levels approach this value. The proteins whose synthesis it activates include Dps, which suppresses Fenton chemistry by sequestering unincorporated iron; Fur, which restores control of iron import; and the Suf system, which helps to maintain the activities of Fe/S enzymes. The determination that MntH also helps suppress Fenton-based protein damage fits this pattern. However, we have not yet identified the specific metabolic failure that causes either the $\text{Hpx}^- \Delta\text{dps}$ mutant or the $\text{Hpx}^- \Delta\text{mntH}$ mutant to stop growing. The suspicion is that an iron-loaded mononuclear-metal enzyme is oxidized and loses activity, thereby plugging a key metabolic pathway. We are currently working to identify such a bottleneck.

Experimental procedures

Reagents

Desferrioxamine (deferrioxamine mesylate), 30% hydrogen peroxide, onitrophenyl- β -D-galactopyranoside (ONPG), acid-hydrolyzed casamino acids (Hy-Case Amino), xanthine, bovine xanthine oxidase, *E. coli* manganese-containing superoxide dismutase, horseradish peroxidase, *E. coli* iron-containing superoxide dismutase, horse heart cytochrome *c*, manganese(II) chloride tetrahydrate, copper(II) sulfate pentahydrate, ferric chloride, ferrous ammonium sulfate hexahydrate, zinc sulfate, and 8-hydroxyquinoline-5-sulfonic acid were from Sigma-Aldrich. Guanidine hydrochloride was obtained from Fisher Scientific. OxyBlot Protein Oxidation Detection Kit (S7150) was from Chemical International. Amplex Red was purchased from Invitrogen.

Bacterial Growth

Luria-Bertani medium (LB) contained (per liter) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl. Defined medium (glucose/amino acids) consisted of minimal A salts (Miller, 1972) supplemented with 0.2% casamino acids, 0.5 mM tryptophan, and 0.2% glucose. When antibiotic selection was needed, media were supplemented with either 100 μ g/mL ampicillin or 20 μ g/mL chloramphenicol.

Anaerobic cultures were grown in an anaerobic chamber (Coy Laboratory Products Inc.) under an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Aerobic cultures were grown with vigorous shaking at 37°C. To ensure that cells were growing exponentially before they were exposed to oxygen, anaerobic overnight cultures of oxygen-sensitive strains were diluted to OD₆₀₀ = 0.005 in fresh anaerobic medium and allowed to grow to OD₆₀₀ ~ 0.15 at 37°C. These cells were then subcultured into fresh aerobic medium to obtain an OD₆₀₀ of 0.005, with or without manganese(II) chloride, and grown aerobically at 37°C. To remove supplemented manganese, cells were washed once and inoculated in the same medium but without MnCl₂.

Strains and strain construction

Strains used in this study are listed in Table 1. All constructions in Hpx⁻ (i.e. *katE katG ahpF*) backgrounds were performed in an anaerobic chamber to ensure that suppressor mutations were not selected during outgrowth. Null mutations were created using the Red recombinase method (Datsenko & Wanner, 2000). Mutations were introduced into new strains by P1 transduction (Miller, 1972). The resultant mutations were confirmed by PCR analysis and, when possible, by enzyme assays.

The chromosomal *mntH(NI)* allele, in which the OxyR binding site is removed and replaced with a *flp* scar sequence, was also generated by the Red recombinase method. These mutations were confirmed by PCR analysis and sequencing. Positions -111 to -75 upstream of the transcription start site were removed in the *mntH(NI)* mutant allele, corresponding to bases 2510803 to 2510839. This deletion removed the OxyR binding site (Kehres *et al.*, 2002a) but did not alter the -10/-35 promoter region or the 5' UTR of the transcript. The plasmid pAA01 expressing the wild-type *mntH* allele and its promoter region (base 2509490-base 2510928) was made using the forward primer 5'-ATCTAAAGCTTGCTATGTTGTGTATGGAAGCTGAAAG and reverse primer 5'-GAATCGGATCCCTACAATCCCAGCGCCGACCCAC with BamHI and HindIII restriction sites. The PCR product was inserted into pBR322 plasmid and confirmed by sequencing.

Single-copy *lacZ* fusions to *sodA* and *mntH* promoter regions were integrated into the lambda attachment site, while the wild-type genes remained at their native positions (Haldimann & Wanner, 2001). The promoter regions were amplified using the forward primer 5'-GAGAGACTAAACGAGCTGTAATACGCC and reverse primer 5'-CATATTCATCTCCAGTATTGTCTGGGCG for *sodA*, and forward primer 5'-ATATCCTGCAGCAACAACGGCAAGTGCCAGTACAAAATG and reverse primer 5'-ATATCGGTACCCATCTTGTGCCTCTAAAACATAGCCTTTG for *mntH*, both designed with PstI and KpnI restriction sites. CRIM plasmids were modified by replacing the kanamycin-resistance cassette with a chloramphenicol-resistance cassette, to permit antibiotic selection under anaerobic conditions, and engineering FLP sites around the chloramphenicol acetyltransferase gene. The *sodA* and *mntH* promoter regions were inserted, and the resultant plasmid constructions were confirmed by sequencing. After chromosomal integration and transduction into recipient strains, the chloramphenicol acetyltransferase gene was removed from mutants, as indicated in table 1, by using the temperature sensitive plasmid pCP20, which was later cured from the strain in question (Datsenko & Wanner, 2000).

Enzyme Assays

Aerobic cultures were grown in defined medium (glucose/amino acids) to an OD₆₀₀ of 0.2, after which cells were centrifuged, washed, resuspended, and sonicated in 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM EDTA. We measured β -galactosidase activity by ONPG hydrolysis (Miller, 1972), and determined total protein content by using the Coomassie blue dye-binding assay (Pierce). SOD activity was measured by the xanthine oxidase-cytochrome *c* method (McCord & Fridovich, 1969). To track the activity of specific SOD isozymes, we measured manganese-containing SOD (MnSOD) in *sodB* mutants and iron-containing SOD (FeSOD) in *sodA* mutants. The fraction of each isozyme that was active in cell extracts was determined after extracts were subjected to partial denaturation and renaturation in the presence of manganese/iron to ensure the full activation of MnSOD or FeSOD proteins. MnSOD was denatured at pH 3.8 in the presence of guanidinium chloride, EDTA, and 8-hydroxyquinoline-5-sulfonic acid and then reactivated by dialysis into neutral pH in buffer containing 0.1 mM MnCl₂. Details follow the published protocol (Kirby *et al.*, 1980) except that 8-hydroxyquinoline-5-sulfonic acid was used instead of 8-hydroxyquinoline and remetallation was accomplished with two changes of 0.1 mM MnCl₂ in 5 mM Tris-HCl buffer at pH 7.8 for 12 h each instead of one. Metals were removed from FeSOD at pH 11 in the presence of EDTA and dithiothreitol, while remetallation was accomplished by dialysis at neutral pH against 0.1 mM ferrous salts and dithiothreitol (Yamakura & Suzuki, 1976), all in an anaerobic chamber (Beyer *et al.*, 1989). For both MnSOD and FeSOD reconstitution, purified MnSOD and FeSOD were used as controls; in both cases, all activity was lost upon demetallation and fully recovered by reconstitution. The periplasmic CuZnSOD is only synthesized in stationary phase, and activity gels confirmed that it did not provide detectable activity under the experimental conditions used here.

Inductively coupled plasma (ICP) measurement of intracellular manganese

One-liter cultures were grown aerobically in defined medium (glucose/amino acids) at 37°C to an OD₆₀₀ of 0.15-0.2. The cells were then centrifuged, washed twice with 200 mL ice-cold 20 mM Tris-HCl/1mM EDTA (pH 7.4), washed once in the same buffer without EDTA, and then resuspended in 4 mL 20 mM Tris-HCl (pH 7.4). Cells were lysed with a French press, and debris was pelleted by centrifugation at 22,000 $\times g$ for 25 min. The metal content of the supernatant was determined with a SCIEX ELAN DRc (Perkin-Elmer), and protein content was determined using the Coomassie blue dye-binding assay (Pierce). To calculate the manganese concentration of intact cells, the degree of dilution that occurred

during lysis was deduced by comparing the protein concentration of the lysate to its known concentration in intact cells (~300 mg/mL, determined previously by isotopic labeling of the cytoplasmic volume, coupled with measurement of cellular protein (Imlay & Fridovich, 1991)).

H₂O₂ concentration measurement

Cells were grown anaerobically in defined medium (glucose/amino acids) to an OD₆₀₀ of ~ 0.15. These cells were then centrifuged, suspended in fresh defined medium at an OD₆₀₀ of 0.005, and grown aerobically at 37°C with vigorous shaking, with or without 50 μM MnCl₂. At regular intervals an aliquot was removed and centrifuged, and H₂O₂ was measured in the supernatant. H₂O₂ was detected using the Amplex Red/horseradish peroxidase method (Seaver & Imlay, 2001a). Fluorescence was then measured in a Shimadzu RF Mini-150 fluorometer.

The rates of H₂O₂ scavenging by whole cells were measured in a similar way, except cells were inoculated aerobically to an OD₆₀₀ of 0.02, and 1 μM H₂O₂ was added to the culture.

Cell Viability

Air-sensitive strains were grown anaerobically in defined medium (glucose/amino acids) to an OD₆₀₀ of ~ 0.15. These cells were then subcultured to an OD₆₀₀ of 0.003 and grown aerobically at 37°C with vigorous shaking. At intervals, aliquots of cells were moved to the anaerobic chamber, diluted in anaerobic LB, and plated on anaerobic LB agar containing 0.2% glucose. Colonies were enumerated after overnight anaerobic incubation at 37°C.

H₂O₂ killing assay

Cells were grown aerobically in defined medium (glucose/amino acids) to an OD₆₀₀ of 0.1-0.15 with or without 100 μM MnCl₂ supplementation. These cells were subcultured to an OD₆₀₀ of 0.025, with or without 100 μM MnCl₂ supplementation. H₂O₂ (2.5 mM) was added, and at different times aliquots were diluted with LB containing catalase, and colonies were enumerated on LB plates after overnight incubation at 37°C.

Protein Carbonylation

Cells were grown anaerobically in defined medium (glucose/amino acids) at 37°C to an OD₆₀₀ of 0.1-0.15. Most strains were diluted to an OD₆₀₀ of 0.005 in freshly made aerobic defined medium (glucose/amino acids), with or without 50 μM MnCl₂ supplementation. Hpx⁻ *mntH* cells, grown without MnCl₂, were subcultured to an OD₆₀₀ of 0.040, since these cells double in biomass only twice before growth ceases. Cells were harvested at an OD₆₀₀ of ~ 0.2. These cells were washed twice with ice-cold 50 mM potassium phosphate buffer pH 7.0, after which they were suspended in 500 μL of the same buffer with the addition of 5 mM DETAPAC. DETAPAC prevents further protein oxidation in extracts. Cells were sonicated, and then protein carbonylation was measured using OxyBlot Protein Oxidation Detection Kit (S7150) (Chemicon International). Protein carbonyl groups were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH) for 15 min in 3% (w/v) SDS. β-mercaptoethanol (1% v/v) was then added to these derivatized samples, after which the samples were subjected to polyacrylamide denaturing gel electrophoresis (4-15% BioRad). Proteins were then transferred to a nitrocellulose membrane (Amersham Hybond™-ECL™ by GE Healthcare) for 60 min at 100 V. The membrane was then incubated with primary antibody specific to the DNP moiety attached to the derivatized proteins. This step was followed by incubation with a horseradish peroxidase antibody conjugate directed against the primary antibody. The membranes were then treated with chemiluminescent substrate (GE Healthcare, Amersham ECL™ Western Blotting

Analysis System) and imaged by exposure to light sensitive films (Amersham Hyperfilm ECL™ chemiluminescence film, GE Healthcare, Buckinghamshire, UK).

Some protein carbonyls were consistently detected in the extracts of cells than had been grown and processed under strictly anaerobic conditions. These carbonyl adducts constitute a background that cannot reflect ROS-mediated oxidative damage and may arise from protein glycation reactions (Adams *et al.*, 2001). This background must be considered when protein carbonylation is used as a proxy for oxidative injury.

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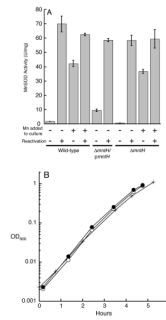
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**Fig 1.**

Unstressed cells do not depend upon manganese. A. In standard medium, manganese import is too slight to activate MnSOD. Where indicated, media were unsupplemented or were supplemented with 50 μ M manganese, and cell extracts were assayed before and after in vitro demetallation/reactivation with manganese. Each strain lacks *sodB*, to permit assay of MnSOD. The strains were AA138 (*mntH*⁺), AA141 (Δ *mntH*), and AA141/pAA01 (Δ *mntH*/*pmntH*). B. Manganese import is unnecessary for rapid growth. Wild-type MG1655 cultured without (open circles) or with (closed circles) 50 μ M manganese supplement, and Δ *mntH* (AA99, plus sign) without manganese.

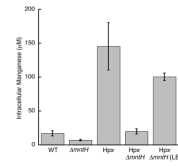


Fig 2.

Total manganese concentration of cells, measured by ICP. Cells were grown in defined medium (glucose/amino acids) or, where indicated, LB medium. Data represents means of three independent cultures. The strains used were MG1655 (wild-type), AA99 (Δ mntH), LC106 (Hpx⁻) and AA30 (Hpx⁻ Δ mntH).

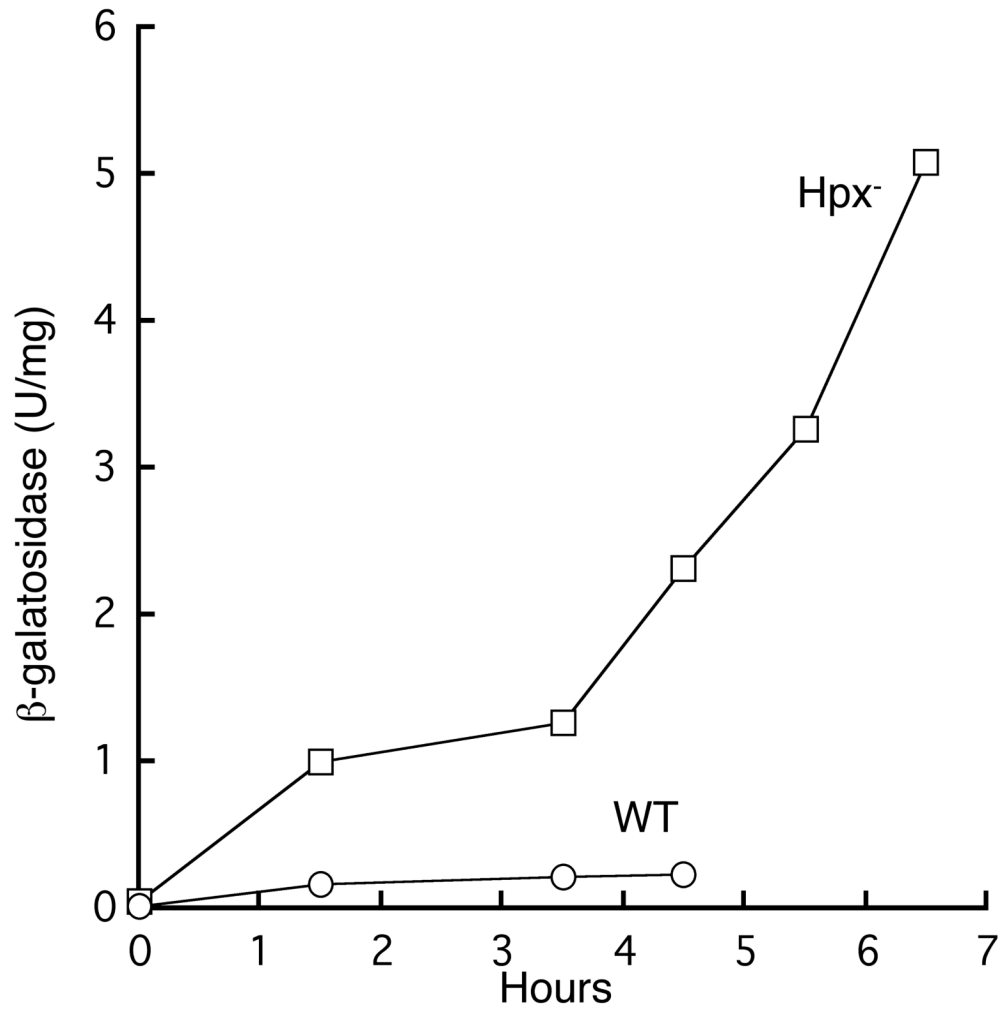
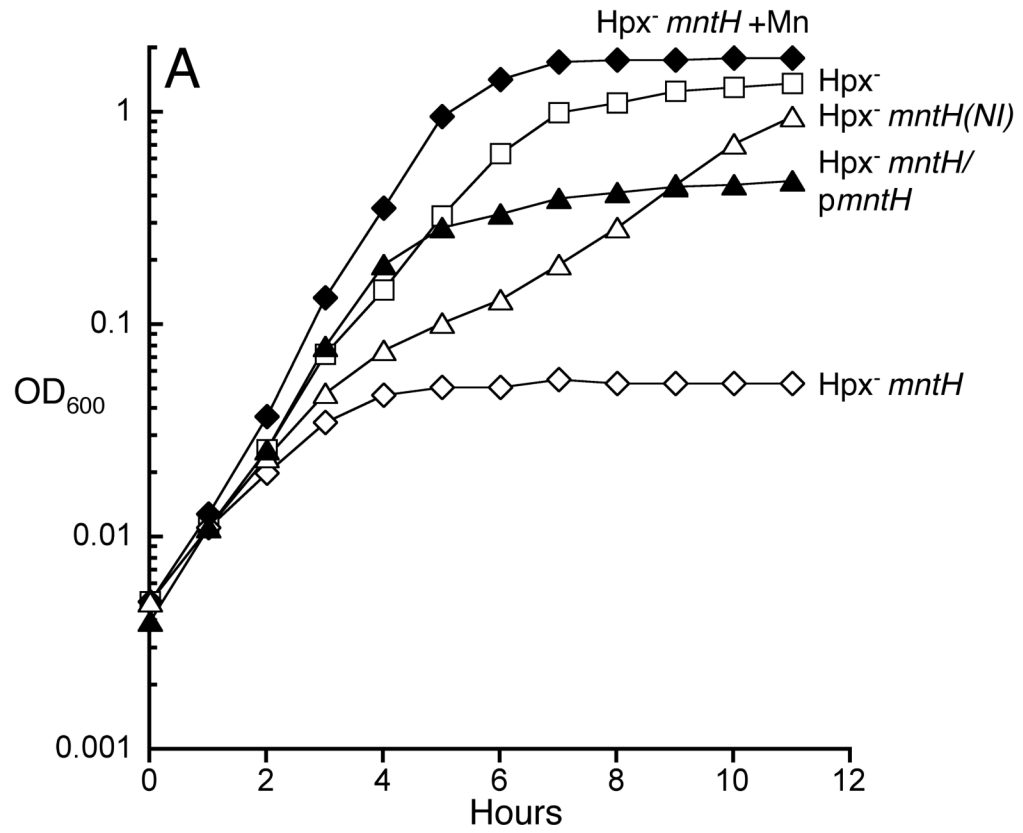


Fig 3. The gene *mntH* is strongly induced in aerobically growing Hpx⁻ cells. Cells bearing a *mntH*⁺-*lacZ* fusion were grown in anaerobic defined medium (glucose/amino acids) and aerated at time zero. At intervals β -galactosidase was assayed. This experiment is representative of four independent replicates. AA183 (wild-type, circles) and AA191 (Hpx⁻, squares).



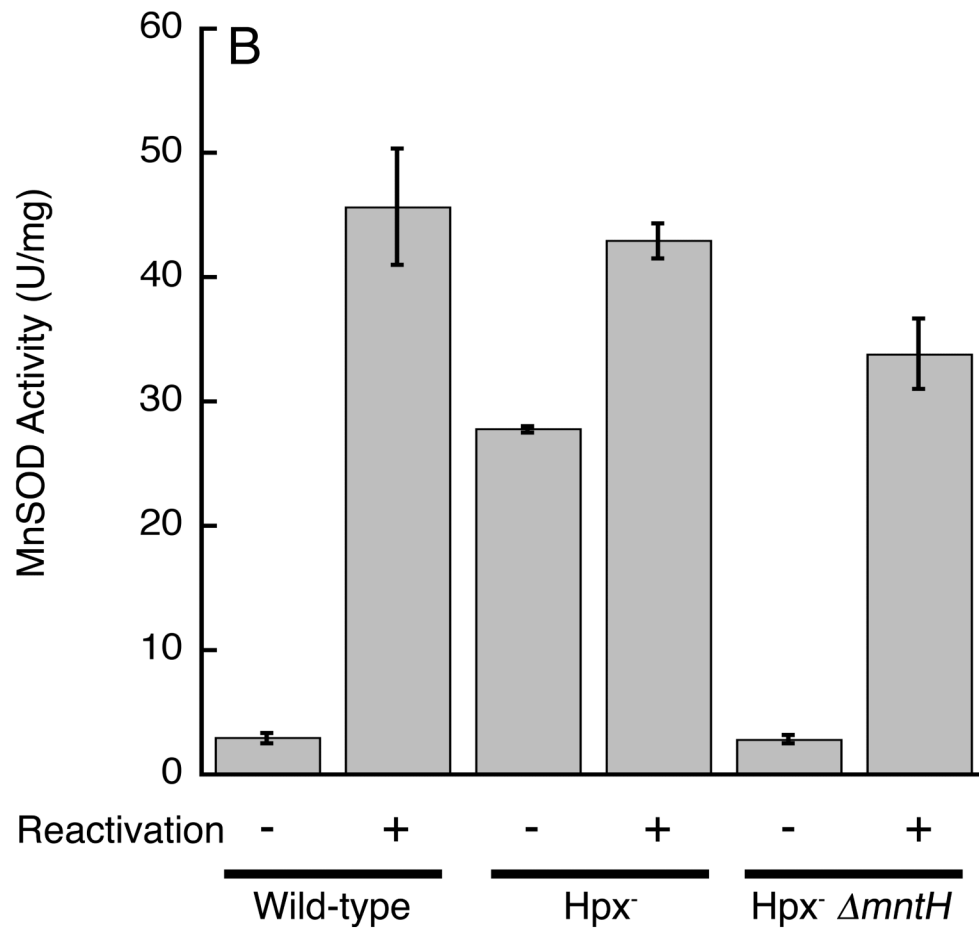


Fig 4. Manganese import is essential for H₂O₂-stressed cells. Cells were grown in defined medium (glucose/amino acids). A. Cells were cultured anaerobically and then aerated at time zero. Where indicated, manganese (50 μM) was included in the culture medium. LC106 (Hpx⁻, open squares), SMV42 (Hpx⁻ Δ*mntH*, open diamonds), SMV42 + MnCl₂ (closed diamonds), SMV42/pAA01 (Hpx⁻ Δ*mntH*/p*mntH*, closed triangles) and AA153 (Hpx⁻ *mntH*(NI), open triangles). B. MnSOD protein is correctly metallated in H₂O₂-stressed cells, due to increased manganese import. Each strain lacks *sodB*, to permit assay of MnSOD. SMV32 (wild-type), SMV29 (Hpx⁻), AA145 (Hpx⁻ Δ*mntH*).

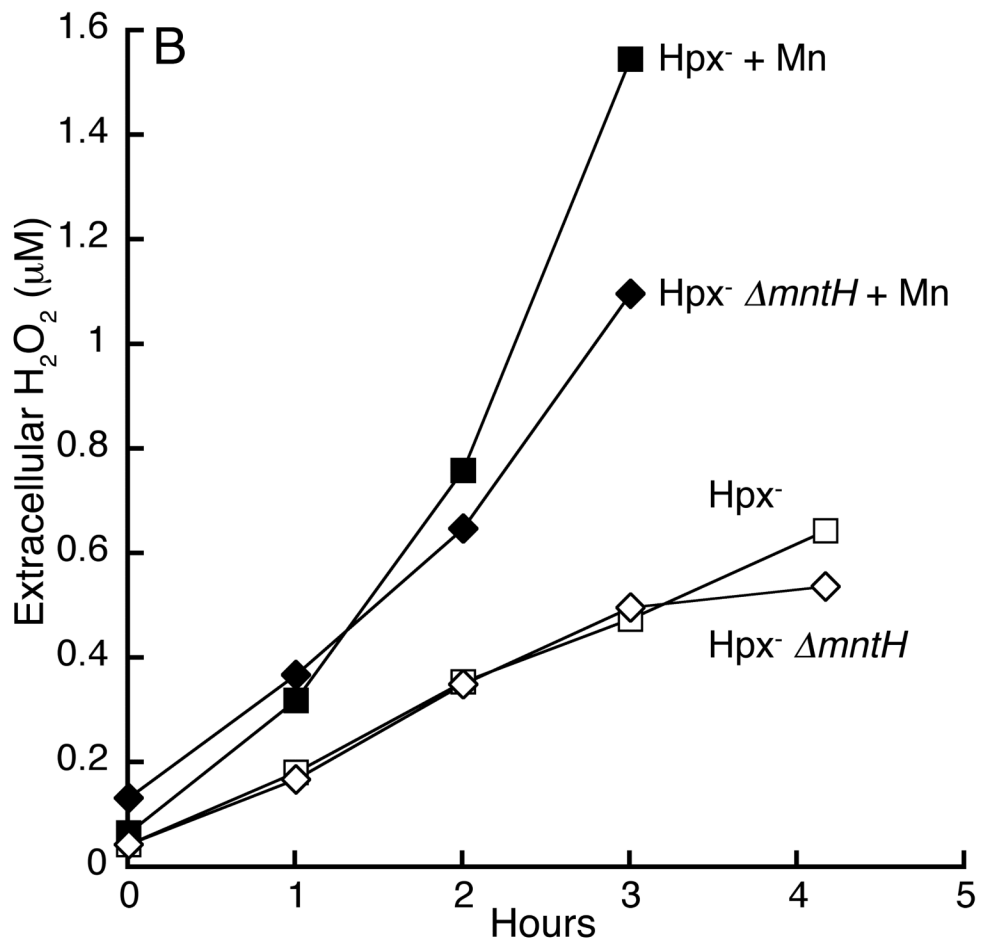
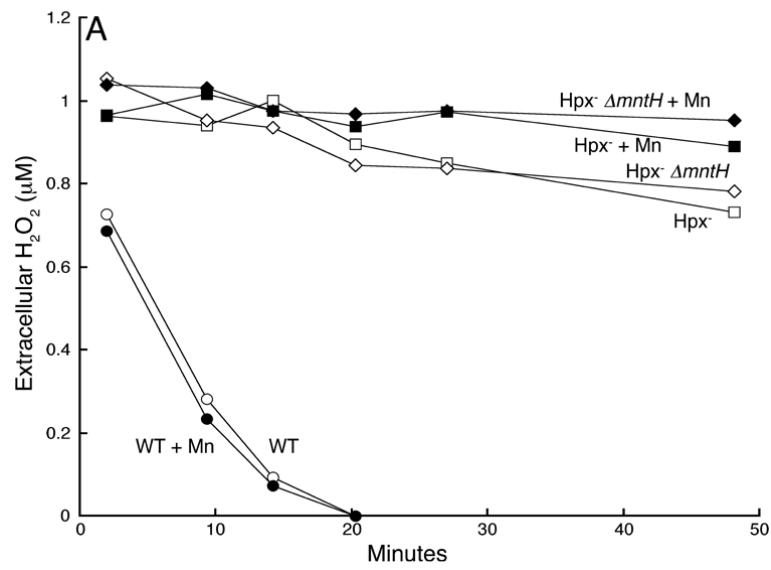


Fig 5. Imported manganese does not scavenge H₂O₂. A. Manganese did not prevent H₂O₂ accumulation by Hpx⁻ and Hpx⁻ Δ*mntH* cells. Cells were grown anaerobically and aerated at

time zero. At intervals the medium was assayed for accumulated H_2O_2 . LC106 (Hpx^- , squares) and SMV42 ($\text{Hpx}^- \Delta\text{mntH}$, diamonds). Filled symbols: 50 μM manganese was included in the growth medium. B. Manganese-supplemented cells did not exhibit significant H_2O_2 -scavenging activity. H_2O_2 (1 μM) was added at time zero to cultures of log-phase cells. Filled symbols: 50 μM manganese was included in the growth medium. MG1655 (open circles), LC106 (Hpx^- , squares), SMV42 ($\text{Hpx}^- \Delta\text{mntH}$, diamonds).

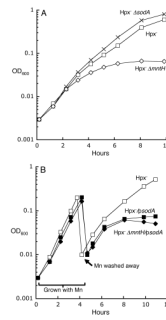


Fig 6.

The primary role of intracellular manganese is not to degrade O_2^- . A. Metallation of MnSOD is not the primary purpose of manganese import. Cells were precultured in anaerobic defined medium (glucose/amino acids) and then aerated at time zero. LC106 (Hpx^- , squares), AA30 ($Hpx^- \Delta mntH$, diamonds) and SMV30 ($Hpx^- \Delta sodA$, X's). B. Overproduction of MnSOD debilitates $Hpx^- \Delta mntH$ cells. Anaerobic cells, with or without the pDT1.5 *sodA* over-expression plasmid, were aerated at time zero in defined medium (glucose/amino acids) containing $100 \mu M MnCl_2$. At the indicated time (arrow), manganese was removed. LC106 (Hpx^- , open squares), LC106/pDT1.5 (closed squares) and SMV42/pDT1.5 ($Hpx^- \Delta mntH$, closed diamonds).

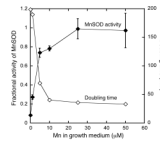
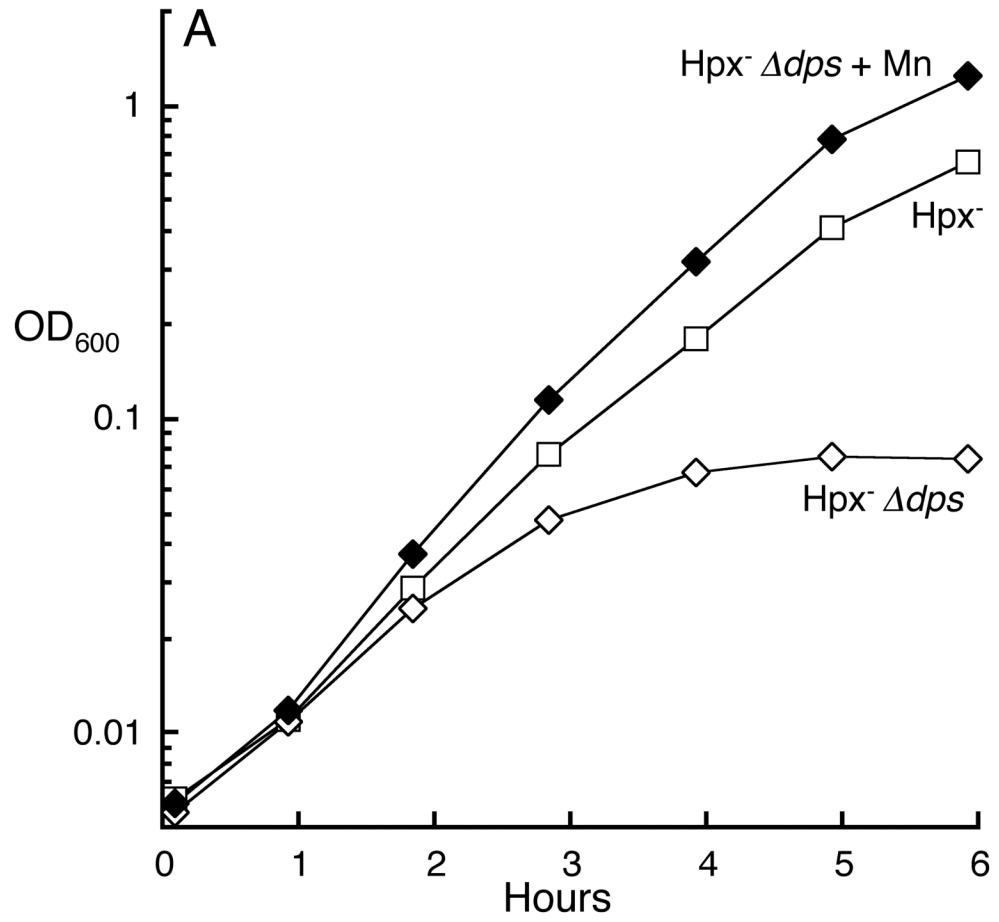


Fig 7.

The amount of manganese needed to protect Hpx⁻ mutants matches the amount needed to metallate enzymes. AA145 (Hpx⁻ Δ *mntH*) cells were precultured anaerobically in defined medium (glucose/amino acids) and then subcultured at time zero into aerobic defined medium (glucose/amino acids) containing the indicated concentration of manganese. Optical density was monitored continuously, and MnSOD activity was measured when the cultures reached an OD₆₀₀ of 0.2. The lowest doubling time, and thus the highest growth rate, for each culture is denoted in the figure.



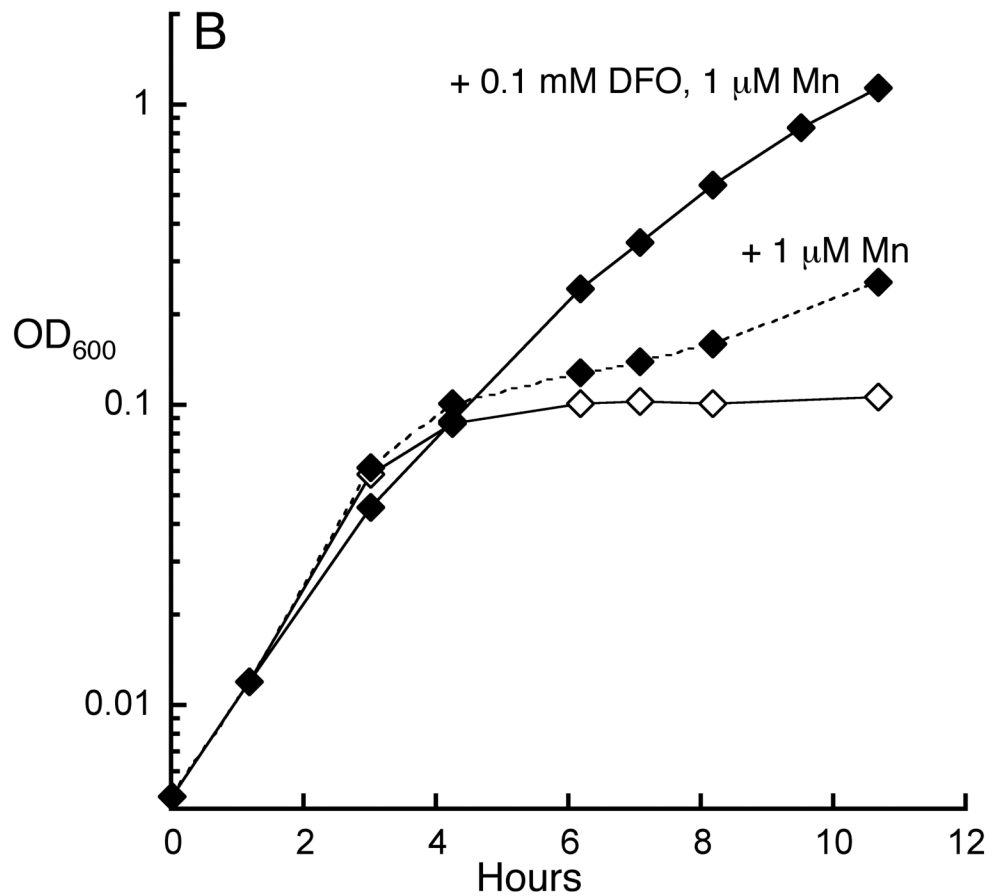


Fig 8. Manganese is required to rescue Hpx^- cells from iron overload. A. Manganese supplementation rescues $Hpx^- \Delta dps$ cells. Cells growing in anaerobic defined medium (glucose/amino acids), +/- 50 μM $MnCl_2$, were aerated at time zero. LC106 (Hpx^- , open squares), SP66 ($Hpx^- \Delta dps$, open diamonds), SP66 grown + $MnCl_2$ (closed diamonds). B. The growth defect of $Hpx^- \Delta mntH$ was suppressed by an iron-specific chelator. Cells (AA30) were grown in aerobic defined medium (glucose/amino acids) without $MnCl_2$ (open diamonds), with 1 μM $MnCl_2$ (dotted line, closed diamonds) or with 1 μM $MnCl_2$ plus 0.1 mM deferoxamine (closed diamonds).

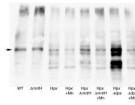


Fig 9.

Imported manganese suppresses oxidative protein carbonylation. Cells were grown in aerobic defined medium (glucose/amino acids) +/- 50 μ M MnCl_2 , and proteins were harvested at an OD_{600} of ~ 0.2. Proteins were derivatized and western blotted as described in experimental procedures. The arrow indicates a non-oxidative carbonylation that was detected even when cells were grown and derivatized anaerobically. The strains used were MG1655 (wild-type), AA99 (Δ *mntH*), LC106 (*Hpx*⁻), AA30 (*Hpx*⁻ Δ *mntH*) and SP66 (*Hpx*⁻ Δ *dps*).

Table. 1

Strain	Genotype	Reference
MG1655	F-wild type	<i>E. coli</i> Genetic Stock Center
LC106	Δ ahpF::kan Δ (katG17::Tn10)1 Δ (katE12::Tn10)1	Seaver and Imlay (2004)
CAG18467	zff-1::Tn10	<i>E. coli</i> Genetic Stock Center
MM2115	mntH9::kan	Kehres and Maguire (2000)
SMV41	mntH9::kan~zff-1::Tn10	P1(CAG18467) × MM2115
SMV42	As LC106 plus mntH9::kan~zff-1::Tn10	P1(SMV41) × LC106
BW25113	lacI rrmB Δ lacZ hsdK Δ araBAD Δ rhaBAD	Datsenko and Wanner (2000)
AA19	BW 25113 Δ mntH2::cat	This work
AA99	MG1655 Δ mntH2::cat	P1(AA19) × MG1655
AA30	LC106 Δ mntH2::cat	P1(AA19) × LC106
JS141	CSH7 Δ ahpCF1::cat Δ (katG17::Tn10)1 Δ katE1::kan	Laboratory Stock
AA118	JS141 mntH9::kan~zff-1::Tn10	P1(SMV41) × JS141
JS148	AB1157 Δ ahpCF1::cat Δ (katG17::Tn10)1 Δ katE1::kan	Laboratory Stock
AA120	JS148 mntH9::kan~zff-1::Tn10	P1(SMV41) × JS148
J1370	MG1655 Δ ahpF::kan	Seaver and Imlay (2001)
AA116	J1370 Δ mntH2::cat	P1(AA19) × J1370
J1364	Δ (katG17::Tn10)1	Seaver and Imlay (2001)
AA142	J1364 Δ mntH2::cat	P1(AA19) × J1364
DH5a pir ⁺	supE44 Δ lacU169 (ϕ 80lacZ Δ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 pir ⁺	Jim Schlauch
SJ99	MG1655 Δ (lacZ1::cat)1	Laboratory Stock
G169	F ⁻ , aroD shiA proA argE zdg-299::Tn10	Grogan and Cronan (1984)
J1132	AB1157 (sodA::Mud PR13)25 (sodB::kan)1- Δ 2	Linn and Imlay (1987)
KK183	J1132 zdg-299::Tn10	P1(G169) × J1132
SMV29	LC106 (sodB::kan)1- Δ 2	Varghese and Imlay (2007)
SMV32	MG1655(sodB::kan)1- Δ 2	Varghese and Imlay (2007)
AA130	MG 1655 Δ (lacZ1::cat)1 att λ ::[pSJ501::sodA'-lacZ ⁺]	This work
AA138	SMV32 Δ (lacZ1::cat)1 att λ ::[pSJ501::sodA'-lacZ ⁺]	P1(AA130) × AA136
AA114	SMV32 Δ mntH2::cat	P1(AA19) × SMV32
AA141	AA114 Δ (lacZ1::cat)1 att λ ::[pSJ501::sodA'-lacZ ⁺]	P1(AA130) × AA137
KCI420	MG1655 (sodA::Mud PR13)25	Laboratory Stock
AA171	MG1655 Δ (lacZ1::cat)1 att λ ::[pSJ501::mntH'-lacZ ⁺]	This work
AA183	MG1655 Δ (lacZ1::cat)1 att λ ::[pSJ501::mntH'-lacZ ⁺]	P1(AA171) × SJ130
SJ108	LC106 Δ (lacZ1::cat)1	Laboratory Stock
AA191	SJ108 att λ ::[pSJ501::mntH'-lacZ ⁺]	P1(AA171) × SJ108
AA147	BW25113 mntH1::cat(NI)	This work
AA153	LC106 mntH1::cat(NI)	P1(AA147) × AA153
SMV42/pAA01	SMV42 with pAA01	This work
AA145	SMV29 Δ mntH2::cat	P1(AA19) × SMV29
LC106/pDT1.5	LC106 with pDT1.5	This work

Strain	Genotype	Reference
SMV42/pDT1.5	SMV42 with pDT1.5	This work
Lem17	MG1655 <i>recA56 srl300::Tn10</i>	Laboratory Stock
XY27	MG1655 plus <i>recA938::cat ΔahpF::kan Δ(katG17::Tn10)1 Δ(katE12::Tn10)1</i>	Park and Imlay (2005)
SP66	LC106 <i>mhpC281::Tn10 lacY1 dps::cat</i>	Park and Imlay (2005)
SMV30	LC106 (<i>sodA::Mud PR13</i>)25	P1(KK183) × LC106
JEM78	BW25113 <i>ΔnrpEF1::cat</i>	Julia Martin
JEM90	LC106 <i>ΔnrpEF1::cat</i>	P1(JEM78) × LC106
AA44	BW25113 <i>Δicd1::cat</i>	This work
AA160	LC106 <i>Δicd1::cat</i>	P1(AA44) × LC106
AA38	BW25113 <i>ΔpgmI1::cat</i>	This work
AA59	LC106 <i>ΔpgmI1::cat</i>	P1(AA38) × LC106
AA28	BW25113 <i>ΔmntR1::cat</i>	This work
AA33	LC106 <i>ΔmntR1::cat</i>	P1(AA28) × LC106
AA79	BW25113 <i>ΔglpX1::cat</i>	This work
AA89	LC106 <i>ΔglpX1::cat</i>	P1(AA89) × LC106
AA94	BW25113 <i>ΔmaeA1::cat</i>	This work
AA110	LC106 <i>ΔmaeA1::cat</i>	P1(AA94) × LC106

Plasmid	Genotype	Reference
pAA01	pBR322 with <i>mntH</i>	This work
pSJ501	pAH125 derivative with <i>cat</i> flanked by <i>flp</i> sites	Soojin Jang
pAA02	pSJ501:: <i>sodA'</i> - <i>lacZ</i> ⁺	This work
pAA03	pSJ501:: <i>mntH'</i> - <i>lacZ</i> ⁺	This work
pDT1.5	pBR322 with <i>sodA</i>	Carlioz and Touati (1986)