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Submicromolar hydrogen peroxide disrupts the ability of Fur protein to control free-iron levels in *Escherichia coli*

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Summary

In aerobic environments, mutants of *Escherichia coli* that lack peroxidase and catalase activities (Hpx^-) accumulate sub-micromolar concentrations of intracellular H_2O_2. We observed that in defined medium these strains constitutively expressed members of the Fur regulon. Iron-import proteins, which Fur normally represses, were fully induced. H_2O_2 may antagonize Fur function by oxidizing the Fur:Fe^{2+} complex and inactivating its repressor function. This is a potential problem, as in iron-rich environments excessive iron uptake would endanger H_2O_2-stressed cells by accelerating hydroxyl-radical production through the Fenton reaction. However, the OxyR H_2O_2-response system restored Fur repression in iron-replete LB medium by up-regulating the synthesis of Fur protein. Indeed, when the OxyR binding site upstream of *fur* was disrupted, Hpx^- mutants failed to repress transporter synthesis, and they exhibited high levels of intracellular free iron. Mutagenesis and bacteriostasis resulted. These defects were eliminated by mutations or chelators that slowed iron import, confirming that dysregulation of iron uptake was the root problem. Thus aerobic organisms must grapple with a conundrum: how to monitor iron levels in oxidizing environments that might perturb the valence of the analyte. The induction of Fur synthesis by the OxyR response comprises one evolutionary solution to that problem.

Keywords

Iron; hydrogen peroxide; Fenton; Fur; OxyR

Introduction

Hydrogen peroxide (H_2O_2) is generated inside aerobic cells when reduced redox enzymes inadvertently transfer electrons to molecular oxygen (Imlay, 2003). While the rate is substantial, the impact is uncertain, because virtually all organisms contain high titers of peroxidases and catalases that rapidly degrade the H_2O_2. The estimated steady-state concentration of H_2O_2 in the model organism *Escherichia coli* is only 20 nM under laboratory growth conditions (Seaver and Imlay, 2001).

Organisms are also exposed to H_2O_2 from external sources. The chemical oxidation of compounds in cell environments can generate low-micromolar H_2O_2, and far higher doses arise from sources such as the oxidative burst of phagocytes, the cycling of redox-active antibiotics, and the excretion of H_2O_2 by lactic acid bacteria. Since H_2O_2 readily crosses membranes, cells that are exposed to these H_2O_2 sources must defend themselves against the stress that ensues.

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Intracellular concentrations of H_2O_2 in excess of 100 nM are sufficient to induce the OxyR stress response system in *E. coli* (Aslund *et al.*, 1999; Seaver and Imlay, 2001), implicitly defining the H_2O_2 level that evolution considers hazardous. It has been a challenge, however, to identify the biomolecules that such a small dose of H_2O_2 can damage. Part of the difficulty is the experimental design. When low concentrations of H_2O_2 are added to cell cultures, scavenging enzymes soon clear the H_2O_2, and so the stress is abbreviated and the impact upon growth processes cannot easily be studied. Millimolar doses can overwhelm these systems and are lethal to most organisms, but they are not physiological.

An alternative approach is to genetically eliminate the scavenging systems, thereby allowing the imposition of low, persistent H_2O_2 stress. We have used hydroperoxidase (Hpx^-) mutants of *E. coli*, which lack its NADH peroxidase and two catalases, for this purpose. When these strains are grown in aerobic medium, H_2O_2 is formed by endogenous processes and equilibrates across the membrane, gradually rising to approximately 1 micromolar both inside and outside the cell. Substantial DNA damage accumulates due to the formation of hydroxyl radicals, as H_2O_2 reacts with unincorporated iron (Park *et al.*, 2005):

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + OH^- + Fe^{3+} \quad [1]$$

In these mutants the H_2O_2 stress would be lethal except for the fact that OxyR triggers the induction of Dps (Almiron *et al.*, 1992; Zhao *et al.*, 2002), an iron-storage protein that partially suppresses DNA damage by sequestering iron in an unreactive form. Thus in aerobic media Hpx^- *oxyR* and Hpx^- *dps* mutants rapidly die (Park *et al.*, 2005). OxyR also induces another dozen or so other proteins, many of whose roles are not yet clear (Zheng *et al.*, 2001a; Zheng *et al.*, 2001b).

Hpx^- mutants also exhibit specific biosynthetic defects, suggesting that H_2O_2 poisons enzymes that belong to particular pathways. In the course of investigating those, we observed that the synthesis of superoxide dismutase is altered. We traced this phenotype to the ability of H_2O_2 to disrupt the function of the iron-homeostatic control protein Fur (Escolar *et al.*, 1999; Braun, 2003). This report describes that effect and a mechanism whereby the OxyR system minimizes its impact.

Results

The *fur* regulon is derepressed in Hpx^- mutants

Hydroperoxidase-deficient (Hpx^-) mutants of *E. coli* lack both catalase and NADH peroxidase activities, and H_2O_2 consequently accumulates up to approximately 1 micromolar in culture media (Seaver and Imlay, 2001). Because H_2O_2 rapidly equilibrates across cell membranes, this concentration closely represents the concentration of H_2O_2 inside the cells. Several growth defects result, including deficiencies in the biosyntheses of aromatic and branched-chain amino acids (Jang and Imlay, 2007). These defects resemble those that are observed in mutants that lack superoxide dismutase (Carlioz and Touati, 1986), and so it was prudent for us to verify that the Hpx^- mutants continue to synthesize normal levels of SOD. Unexpectedly, using several independent *E. coli* K-12 lineages, we found that Hpx^- mutants actually contained substantially more SOD than did wild-type cells: 11.3 +/− 0.9 vs. 3.0 +/− 0.4 U/mg.

Activity gels revealed that the increase in total SOD activity was due to elevated levels of the manganese-containing SOD (MnSOD, encoded by *sodA*) (Fig. 1A). Strikingly, iron-containing SOD (FeSOD, *sodB*) was almost entirely absent.

The level of each isozyme can be quantified in extracts prepared from mutants that lack the other isozyme. The data indicated that in Hpx^- mutants the MnSOD was induced approximately seven-fold above the levels that were present in Hpx^+ strains (Fig. 1B). The FeSOD was almost undetectable.

MnSOD synthesis is controlled by at least four regulatory proteins. Transcription of the *sodA* gene is stimulated by the SoxRS system when cells are treated with redox-cycling drugs that trigger intracellular superoxide formation (Hassan and Fridovich, 1977; Ding and Demple, 1997; Gaudu *et al.*, 1997), and other labs have shown that millimolar levels of H_2O_2 can also activate this system (Manchado *et al.*, 2000; Zheng *et al.*, 2001b). However, another SoxRS-inducible enzyme, glucose-6-phosphate dehydrogenase, was not induced in Hpx^- mutants, and the MnSOD level in Hpx^- mutants did not diminish when we introduced a *ΔsoxRS* null mutation (data not shown). Since two other regulators of *sodA* transcription--Fnr and Arc (Hassan and Sun, 1992; Compan and Touati, 1993)--should be deactivated in aerobic conditions, we suspected that the one remaining *sodA* regulator--the Fur repressor--might be inactivated in Hpx^- mutants.

When *E. coli* is replete with iron, the Fur repressor binds Fe^{2+} and acquires activity as a repressor both of *sodA* and of multiple genes encoding iron-import systems (reviewed in Escolar *et al.*, 1999, and Braun, 2003). Indeed, the introduction of a *fur* mutation into a wild-type strain increased MnSOD levels several-fold, while it did not further elevate MnSOD levels in the Hpx^- strain (data not shown). This result supported the idea that endogenous H_2O_2 derepresses the Fur regulon when Hpx^- mutants are grown in defined medium.

The Fur protein indirectly controls FeSOD synthesis as well (Masse and Gottesman, 2002; Geissmann and Touati, 2004). When the cell is replete with ferrous iron, Fe^{2+}-metallated Fur protein represses the synthesis of the RyhB small RNA, which would otherwise trigger the degradation of *sodB* mRNA. In combination with the direct repression of MnSOD by Fur, this control mechanism directs the cell to synthesize FeSOD when it perceives that it has sufficient iron to activate it, and to synthesize MnSOD only when iron levels are inadequate. The addition of a *ryhB* null mutation restored the synthesis of FeSOD to Hpx^- mutants (Fig. 1A, lane 4; Fig 1B, lane 7). It had no effect upon wild-type cells. Thus the altered regulation of SOD synthesis indicated that the Fur repressor was inactive in Hpx^- mutants.

Intracellular hydrogen peroxide stimulates transcription of iron-import systems

If the Fur repressor is inactivated by H_2O_2, then the iron-uptake systems that Fur controls should also be derepressed. This prediction was tested by monitoring the expression of the *iuc* operon, which provides some *E. coli* strains with an additional siderophore biosynthetic pathway. Like other iron-transport systems, expression of *iuc* is directly repressed by the Fur:Fe^{2+} complex. An *iucC::lacZ* fusion was introduced into the Hpx^+ and Hpx^- strains, and expression was monitored in the same defined medium that was used in the studies of SOD synthesis. In the wild-type strain the fusion was only expressed when dipyridyl, an iron chelator, was added to the culture. However, the fusion was fully induced in the Hpx^- strains even without the chelator (Fig. 2A). This result confirms that the Fur regulon is derepressed.

Under the conditions of these experiments, the intra- and extracellular H_2O_2 concentration rose to only 0.5–1.0 micromolar. An earlier study showed that these levels of H_2O_2 are sufficient to rapidly oxidize chelated ferrous iron through the Fenton reaction (Park *et al.*, 2005). Because Fur repressor activity depends upon maintaining bound iron in the ferrous state, the oxidation of the Fur:Fe^{2+} complex provides a likely mechanism whereby H_2O_2 might derepress the Fur regulon (see Discussion).

The induction of *fur* by the OxyR system minimizes growth defects

The induction of iron-uptake systems seems like a misguided response for H_2O_2-stressed cells, since the rapid import of iron could make cells vulnerable to Fenton chemistry. Indeed, in Hpx$^-$ strains the OxyR response to H_2O_2 triggers the overexpression of the Fur protein, perhaps in an effort to shut the Fur regulon back off (Zheng *et al.*, 1999). The *iucC::lacZ* data indicate that in defined medium this effort fails, presumably because iron availability is limited. The addition of 100 μM ferrous sulfate restored full repression (data not shown).

In complex medium, however, iron is more abundant and the gene fusion is fully repressed (Fig. 2B). Hpx$^-$ *oxyR* mutants are inviable in aerobic medium (Park *et al*., 2005. Therefore, to test whether the repression of *iucC* depends upon the OxyR-mediated induction of *fur*, we created a mutant strain in which the OxyR binding site upstream of the *fur* gene was selectively deleted (Materials and Methods). The resultant non-inducible *fur* allele (*fur^{NI}*) was fully functional in the absence of H_2O_2 stress, as Fur repressed *iucC::lacZ* in Hpx$^+$ cells unless chelators were added to starve the cell for iron (data not shown). In addition, Northern-blot analysis confirmed that the basal transcription of *fur* was unaffected (Fig. 2). However, when the *fur^{NI}* allele was transduced into the Hpx$^-$ background, the *iucC::lacZ* fusion was significantly derepressed in LB medium, being expressed at a level between the repressed level of the Hpx$^-$ Fur$^+$ strain and the fully-induced level of strains that are starved for iron (Fig. 2B). Therefore, during H_2O_2 stress the OxyR-dependent induction of Fur synthesis is needed to keep the Fur regulon repressed.

Strikingly, Hpx$^-$ strains exhibited growth defects that increased in parallel with their inability to synthesize Fur repressor (Fig. 3). Upon dilution into aerobic medium, all strains grew well for several generations. Strains with a wild-type *fur* allele grew continuously. In contrast, after two hours of growth, strains that lacked the OxyR binding site upstream of *fur* exhibited a prolonged lag and some loss of viability. Finally, strains bearing a *fur::kan* null allele lagged even longer, and their viability declined tenfold during the lag. The eventual outgrowth of these mutants may be due to the OxyR-mediated induction and action of Dps, an iron-sequestration protein, as Hpx$^-$ *dps* mutants do not recover from a growth lag (Park *et al*., 2005). In all cases the growth defects were fully eliminated by the addition of a plasmid containing the wild-type *fur* allele but not by a control vector (data not shown).

Cell-permeable iron chelators can prevent the intracellular formation of hydroxyl radicals by binding iron in a redox-inactive form (Imlay *et al.*, 1988). In fact, the addition of desferrioxamine to the growth medium eliminated the growth lag of the Hpx$^-$ *fur* null mutant (Fig. 3A, dashed line) and the Hpx$^-$ *fur^{NI}* mutant (not shown). Cell death was also prevented (not shown).

Hydroxyl radicals will damage whatever biomolecules they encounter, but the target of greatest consequence is typically DNA. One morphological consequence of DNA damage in *E. coli* is the appearance of cell filaments, which arise when this bacterium cannot replicate its DNA and therefore postpones septation. Hpx$^-$ mutants exhibit some filamentation (Fig. 4). Filamentation was more extensive during the growth lab of Hpx$^-$ *fur^{NI}* mutants, suggesting that derepression of the Fur regulon accelerated DNA damage. Filaments were not formed when desferrioxamine was added.

To independently test whether DNA damage was occurring, mutation rates were measured in wild-type (Hpx$^+$ Fur$^+$), Hpx$^-$ Fur$^+$, Hpx$^-$ *fur^{NI}*, and Hpx$^-$ *fur::kan* strains. Cultures were grown into exponential phase anaerobically, shifted into aerobic medium for four hours, and then plated on anaerobic trimethoprim plates for quantitation of the *thyA* mutations that had occurred during the period of aeration. Wild-type cells cultures contained 1×10^{-6} *thyA* mutants per viable cell. The Hpx$^-$ mutant had 9-fold more mutants, whereas the rates were

110 and 650-fold higher in the Hpx$^-$ *fur*^{NI} and the Hpx$^-$ *fur::kan* strains, respectively. These results suggest that derepression of the *fur* regulon substantially exacerbates the high rate of DNA damage that already occurs in Hpx$^-$ mutants.

OxyR must induce *fur* to avoid excessively high levels of intracellular iron

The unincorporated iron that catalyzes DNA damage can be quantified inside intact cells by EPR spectroscopy (Woodmansee and Imlay, 2002). Desferrioxamine is added to aerobic cultures, and intracellular desferrioxamine-iron chelates exhibit a signal at 4.3 G. Desferrioxamine captures iron that is loosely bound to biomolecules, including DNA; however, desferrioxamine does not appear to remove iron that has been incorporated into metalloproteins.

The unincorporated (free) iron levels were substantially higher inside Hpx$^-$ mutants than wild-type cells (Fig. 5). Consistent with our expectation, levels were particularly high in Hpx$^-$ strains that carry the *fur*^{NI} allele.

To establish a direct connection between excessive iron import and the growth defects of the *fur* mutants, we tested whether the defects could be avoided if import were slowed. EDTA limits the availability of iron for the transport systems, and it almost completely suppressed the problems, even for Hpx$^-$ mutants bearing the *fur* null allele (Fig. 6). The same result was achieved by the introduction of a *tonB* mutation. The TonB protein powers iron uptake through several high-affinity systems that Fur would normally repress in this medium (Wiener, 2005). We deduce that H_2O_2 stress endangers cells by inactivating Fur-dependent repression of TonB-dependent iron import systems and that OxyR induces Fur in order to partially correct this situation.

Discussion

Organisms monitor intracellular Fe^{2+} levels to ensure iron sufficiency. Because hydrogen peroxide efficiently converts ferrous iron to its ferric form, bacteria are vulnerable to the error of mistaking the presence of H_2O_2 for the absence of iron. This misperception may have disastrous consequences, if their response is to increase iron uptake. To avoid this error, *E. coli* has evolved the OxyR system, which detects H_2O_2 and attempts to correct the cell in two ways: by increasing the cellular titer of Fur protein, in hopes that the amount of Fur:Fe^{2+} complex will reach a level that turns import systems back off; and by synthesizing large amounts of Dps, an iron-storage protein that sequesters the ferrous iron that uptake systems may inappropriately import. During periods of chronic, sub-micromolar oxidative stress, both strategies are critical, as Hpx$^-$ *oxyR* (Park *et al.*, 2005) and Hpx$^-$ *fur*^{NI} mutants both exhibit high rates of mutagenesis and toxicity.

The reactivity of the Fur:Fe^{2+} complex with H_2O_2 is a simple example of Fenton chemistry. Initial investigations of the Fenton reaction were conducted by chemists, who worked at acidic pH in order to ensure the solubility of the ferrous iron. Under those conditions they determined the rate constant of the reaction to be 76 M^{-1} s^{-1} (Walling, 1975). This value is relatively low, and when it entered the biological literature some workers inferred that, at physiological levels of H_2O_2, Fenton chemistry was unlikely to occur quickly enough to comprise a significant threat (discussed by Halliwell and Gutteridge, 1990). However, when ferrous iron is complexed by anionic ligands--either hydroxide anion in neutral-pH solutions, or by protein or nucleic acid ligands--the reaction rate is elevated by orders of magnitude (Rush *et al.*, 1990; Park *et al.*, 2005). By extrapolation from measurements made at lower temperatures, we estimate that at neutral pH the rate constant for the Fenton reaction with hexaqueous or ATP-bound iron is 20,000–30,000 M^{-1} s^{-1} at 37° C. Therefore, when 0.5 μM H_2O_2 is present, as in the experiments reported here, the half-time of Fe^{2+}

oxidation will be about 1 min. Thus cell damage through Fenton chemistry is a real hazard, including the DNA damage. These doses of H_2O_2 also rapidly inactivate some enzymes that contain [4Fe-4S] clusters, through an analogous reaction between H_2O_2 and a solvent-exposed iron atom (Jang and Imlay, 2007). Strikingly, at this point each of the phenotypes of Hpx^- mutants has been tracked to reactions between H_2O_2 and iron.

These studies affirm a long-standing hypothesis: aerobic organisms evolved H_2O_2-scavenging enzymes to avoid being poisoned by endogenous H_2O_2. However, their evolution did not solve the problem of oxidative stress completely. Because the cell membrane is a weak barrier to H_2O_2, it does not permit a large outside-to-inside concentration gradient (Seaver and Imlay, 2001); therefore, wild-type cells need encounter only ~ 5 μM H_2O_2 in their environment for the intracellular levels to rise to the same 1 μM dose that damaged DNA, inactivated enzymes, and disrupted iron homeostasis in the Hpx^- mutants. It is for this reason that H_2O_2 is a weapon of choice among competing organisms.

If the OxyR system evolved to defend cells against external H_2O_2, then even scavenger-proficient (Hpx^+) cells that bear the fur^{NI} allele should exhibit sensitivity to H_2O_2. No impact was expected or observed under usual experimental conditions, as moderately dense cultures scavenge micromolar H_2O_2 from the media very rapidly (data not shown). Therefore we measured the outgrowth of fur^{NI} and *Δfur* (Hpx^+) strains when they were diluted to 10^3 cfu/ml into LB medium spiked with micromolar H_2O_2. In all trials, the recovery of *Δfur* mutants lagged substantially, as they achieved a measurable absorbance approximately three generations after wild-type cells. In each of five trials the fur^{NI} mutants trailed the wild-type strain by an average of 0.5 generations--but the variation among triplicate samples was too large to provide statistical significance. We have not identified the source of the data dispersion, so at present this result must be regarded as uncertain. It is probable that bacteria confront protracted H_2O_2 stress in natural environment, but the difficulty of replicating this exposure is the reason that we employed Hpx^- strains in this study.

Interestingly, Lee and Helmann have shown that *Bacillus subtilis* has exploited the reactivity of protein-bound ferrous iron to create an alternative mechanism of H_2O_2 sensing (Lee and Helmann, 2006). PerR is an H_2O_2 sensor that plays the same role in *B. subtilis* as does OxyR in *E. coli*, as it regulates many of the same defensive proteins, including homologues of catalase, Ahp, and Dps. However, PerR structurally resembles Fur, and it binds ferrous iron in a similar coordination environment. As H_2O_2 levels rise, the oxidation of the bound iron atom inactivates the repressor activity of the protein, allowing induction of the genes that it otherwise represses. In PerR, the Fenton reaction triggers oxidation of histidyl ligands of the iron cofactor. It is not yet clear whether upon oxidation the *E. coli* Fur protein is similarly modified. In any case, the ferric ion that is produced in Fur is expected to dissociate from the protein, as the failure of ferric iron to adhere to the binding site is the reason that many workers have used stable Mn^{2+} as a cofactor during aerobic in vitro experiments.

The mammalian IRE-binding protein may represent an evolutionary adaptation that avoids the Fur problem and allows cells to distinguish oxidative stress from iron deficiency (reviewed by Cairo *et al.*, 2002). This aconitase-like protein has an exposed [4Fe-4S] cluster that cannot be formed when iron is scarce; in that situation the apoprotein binds to mRNAs, stabilizing those that encode iron-uptake proteins and destabilizing those that encode iron-storage proteins. As mentioned, H_2O_2 can directly oxidize and destabilize such clusters. However, when [4Fe-4S] clusters are oxidized, a [3Fe-4S] form, rather than an apoprotein, is generated (Brown *et al.*, 2002; Jang and Imlay, 2007). Experiments to date suggest that the residual cluster is fairly stable and that it continues to occupy the cleft that must be empty for IRE-binding protein to bind RNA. When iron is available, the cluster is directly

remetallated to the native [4Fe-4S] form (Djaman *et al.*, 2004). As a result, the oxidized protein should not behave like the apoprotein, and the presence of H_2O_2 would not be mistaken for the absence of iron.

Experimental procedures

Chemicals and strains

Desferrioxamine (deferoxamine mesylate), diethylenetriaminepentaacetic acid (DETAPAC), dipyridyl, o-nitrophenyl B-D-galactopyranoside (ONPG), casamino acids, xanthine, xanthine oxidase, ferric chloride, thymidine, and trimethoprim were from Sigma. Nitroblue tetrazolium was from Fisher, and manganese chloride was from Aldrich.

Strains

Strains used in this study are listed in Table 1. All constructions in Hpx^- (i.e., *katE katG ahp*) backgrounds were performed in an anaerobic chamber to ensure that suppressor mutations were not selected during outgrowth. Transductions and matings were by standard procedures (Miller, 1972). Mutant alleles were selected by the associated antibiotic resistance. Mutations in *soxS* and *ryhB* were verified by PCR analysis; in *sodA* and *sodB*, by activity gel (Beauchamp and Fridovich, 1971); in *fur*, by the co-transduced *cam* allele. Transconjugates that received the pColV plasmid bearing the *iucC::lacZ* allele were identified by the induction of B-galactosidase when grown in LB medium containing 1 mM dipyridyl.

To create plasmids overexpressing the wild-type *fur* allele, primers that flank the *fur* gene and its promoter region (709,200–710,400) were designed with EcoRI and XbaI sites. The PCR product was inserted into the pWKS30 plasmid. The chromosomal fur^{NI} allele, in which the OxyR binding site is removed and replaced with an *flp* scar sequence, and the *tonB* mutant were generated by standard methods (Datsenko and Wanner, 2000). The resultant mutations were confirmed by PCR preparation and sequencing. In the fur^{NI} mutant allele bases 710,094–710,129 have been removed, corresponding to positions −43 to −78 upstream of the transcriptional start site. The deletion removes the OxyR binding site (Zheng *et al.*, 1999) but does not alter the −10/−35 promoter region or the 5′ UTR of the transcript.

Growth curves and survival of strains

Defined medium consisted of minimal A salts (Miller, 1972) supplemented with 0.2% glucose and 0.2% casamino acids, and complex medium consisted of Luria broth (LB) (Miller, 1972). Overnight cultures were grown in anaerobic medium inside an anaerobic chamber (Coy Laboratory Products, Inc.). They were then diluted to approximately 0.010 OD_{600} in fresh anaerobic medium and grown in the chamber for 3–4 generations. For studies of aerobic stress, these exponentially growing cells were then subcultured either to an initial OD_{600} of 0.001 for experiments in LB medium or to an OD_{600} of 0.015 for studies in defined medium. Under these conditions Amplex red/HRP methods (Seaver and Imlay, 2001) show that the concentration of H_2O_2 in defined medium gradually rises in several hours to about 0.8 micromolar ((Park *et al.*, 2005) and data not shown). The concentration of H_2O_2 in LB medium could not be directly determined, because components of the medium interfere with the H_2O_2 assay; however, data suggest that photochemical reactions between room light and chromophores in the medium gradually create 1–5 micromolar H_2O_2 (unpublished data).

Cells were photographed using a phase contrast microscope after six hours of growth in aerobic LB medium. The viability of aerobic cultures was monitored by transferring aliquots

into the anaerobic chamber. The cultures were then diluted in LB medium and plated in anaerobic top agar on anaerobic LB plates. Colonies were counted after 24 hrs.

Enzyme assays

Aerobic cultures were grown in defined medium to an OD_{600} of 0.5. For measurements of SOD activity, extracts were then prepared and assayed by the xanthine/xanthine oxidase method (McCord and Fridovich, 1969) or visualized on activity gels (Beauchamp and Fridovich, 1971) by standard procedures. In some cases, extracts were first subjected to partial denaturation and renaturation in the presence of metals to ensure the full activation of metal-specific SOD isozymes (Privalle and Fridovich, 1988). B-galactosidase activity was measured in extracts prepared in the same buffer (Miller, 1972). Total protein was determined using a dye-binding assay (Pierce).

RNA analysis

Anaerobic cultures in LB medium were diluted into aerobic LB medium and grown for 1 hour. RNA was extracted, isolated, and analyzed by Northern blot as described (Masse and Gottesman, 2002). A biotinylated DNA probe containing the *fur* gene sequence 5′-AGCGTTACTTTCACCCCAGCTTTCTTTAGGGCGGTATTGT- 3′ was used to probe for the *fur* RNA transcript.

EPR measurements

Unincorporated iron was measured by EPR spectroscopy (Woodmansee and Imlay, 2002). Cells were grown in 1 liter of freshly autoclaved LB aerobically as described above. MG1655 was harvested at an OD_{600nm} of 0.1, while Hpx^{-} strains were harvested 4 hours into the growth curve. Cells were pelleted by centrifugation at 8,000 rpm for 5 mins at 4°C. The cell paste was resuspended in 9 mls of LB, and 1 mL of 0.1 M DETAPAC (pH 7.0) and 1 mL of 0.2 M desferrioxamine (pH 8.0) were added sequentially. DETAPAC is a strong cell-impermeant chelator that blocks further iron import, while desferrioxamine is a cell-penetrating iron chelator that captures unincorporated intracellular ferrous iron and triggers its oxidation to the EPR-active ferric form. At the millimolar doses of desferrioxamine that were used in these experiments, its import does not depend upon the Fhu system and therefore is not affected by the status of the *fur* gene. The culture was then incubated aerobically at 37°C for 15 mins. The cells were then centrifuged at 4° C, and the pellet was washed twice with ice-cold 20 mM Tris-Cl (pH 7.4) and then resuspended in 200 μl of the same ice cold buffer containing 10% glycerol. Most of the dense cell suspension (200 μl) was added to the EPR tube and promptly frozen on dry ice. Another aliquot was diluted, and its absorbance was measured. The use of a factor that transforms absorbance into cell volume (1 ml of 1 OD cells comprises 0.47 μl cytoplasmic volume (Imlay and Fridovich, 1991)) allowed subsequent calculations of intracellular iron concentrations. Iron standards were prepared and EPR measurements were conducted according to previous methods.

Measurement of mutagenesis

Cells were grown and inoculated into aerobic LB medium as described above; parallel cultures were grown in anaerobic LB medium. Four hours into the growth curve, aliquots of cultures were diluted into minimal A salts in the anaerobic chamber. They were then aliquotted into F-top agar containing catalase and either thymidine (to determine total viable cells) or thymidine plus trimethoprim (TMP; to determine *thyA* mutants) and spread onto defined-medium plates. Colonies were quantified after 24 hrs. Subsequent experiments verified that trimethoprim-resistant colonies could replate on defined medium only if thymidine was supplied, consistent with a *thyA* genotype (Miller, 1972).

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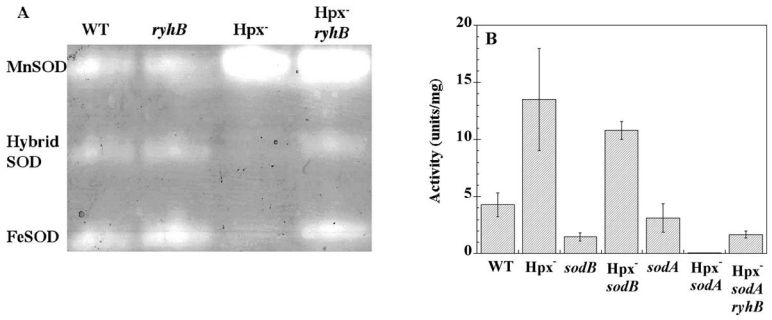

Figure 1. MnSOD and FeSOD activities in Hpx⁻ mutants

(A) SOD activity gels. Congenic strains were grown aerobically in defined medium to an OD_{600} of 0.5. Lane 1: Wild type (MG1655); lane 2: *ryhB* mutant (EM1456); lane 3: Hpx⁻ mutant (LC106); lane 4: Hpx⁻ *ryhB* mutant (SMV36). (B) SOD activities. Extracts were prepared as in panel A, and SOD activity was determined by the xanthine-xanthine oxidase method. MG1655 (wild type), LC106 (Hpx⁻), SMV32 (*sodB*), SMV29 (Hpx⁻ *sodB*), KCI420 (*sodA*), and SMV31 (Hpx⁻ *sodA*), and SMV40 (Hpx⁻ *sodA ryhB).*

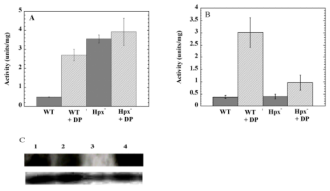

Figure 2. Expression of the Fur regulon in Hpx⁻ mutants

(A) Aerobic defined medium. (B) Aerobic LB medium. WT: wild-type for Hpx. DP: Dipyridyl added. The strains were MS100 (Hpx$^+$ *iucC::lacZ*), MS102 (Hpx$^-$ *iucC::lacZ*), and SMV59 (Hpx$^-$ *iucC::lacZ fur^{NI}*). The results represent the averages of three independent experiments. C) Levels of *fur* RNA transcript (top gel) and 5S RNA (bottom gel). Lane 1: MG1655 (WT, Hpx$^+$); lane 2: SMV55 (Hpx$^+$ *fur^{NI}*); lane 3: KCI402 (Hpx$^+$ *fur::kan*); lane 4: LC106 (Hpx$^-$).

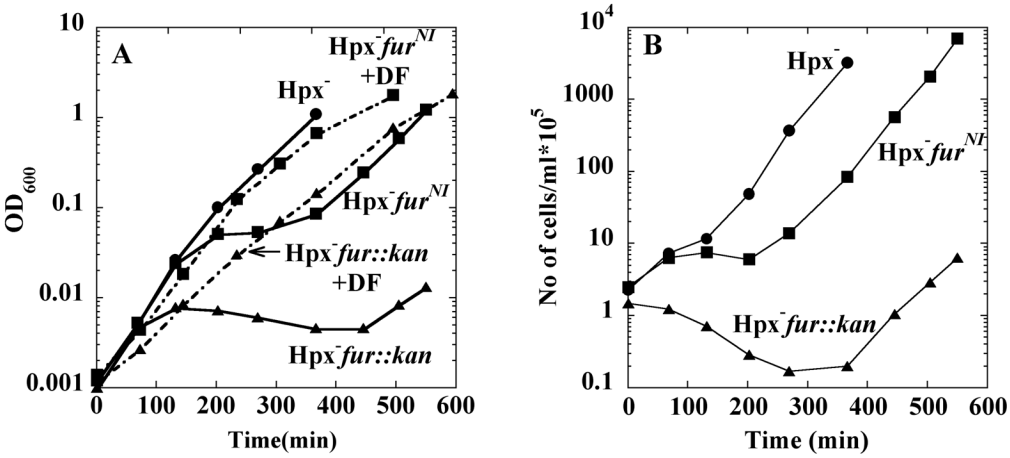

Figure 3. Growth behavior of Hpx⁻ strains in LB medium

(A) Growth after strains were subcultured into LB medium at time zero. Hpx⁻ mutant (LC106, circles), Hpx⁻ *fur*^{NI} (SMV57, squares), Hpx⁻ *fur::kan* (KCI323, triangles). Dashed lines: 2 mM desferrioxamine was included in the medium of the Hpx⁻ *fur*^{NI} (squares) or Hpx⁻ *fur* (triangles) mutants. (B) Number of viable cells during the experiment of panel A. Viability was determined at time points by anaerobic plating and colony enumeration.

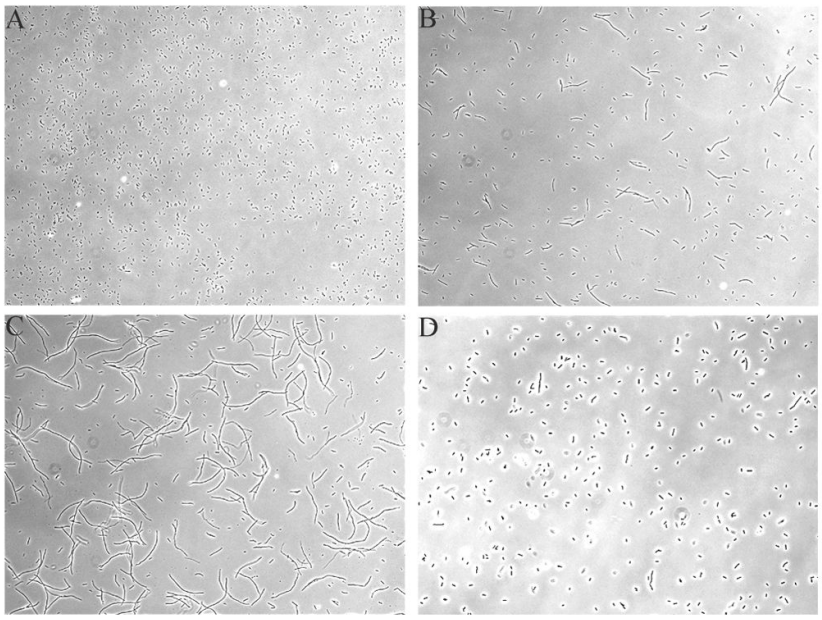

Figure 4. Filamentation of Hpx^{-} *fur*^{NI} cells

Photographs were obtained 6 hrs into the aerobic growth period. (A) MG1655 (wild type), (B) LC106 (Hpx^{-}), (C) SMV57 (Hpx^{-} *fur*^{NI}), and (D) SMV57 (Hpx^{-} *fur*^{NI}) with 20 mM desferrioxamine in the medium. KCI323 (Hpx^{-} *fur::kan*) exhibited morphologies similar to that of panels C and D.

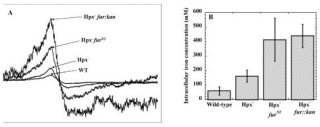

Figure 5. Intracellular concentrations of unincorporated iron

Strains were grown in aerobic LB medium for four hours, and intracellular iron concentrations were measured using EPR spectroscopy. (A) Representative EPR spectra of the strains, normalized to cell density. (B) Quantification of the intracellular iron concentrations. The values are the averages of three independent experiments. The strains used were MG1655 (wild type), LC106 (Hpx$^-$), SMV57 (Hpx$^-$ *fur*^{NI}) and KCI323 (Hpx$^-$ *fur::kan*).

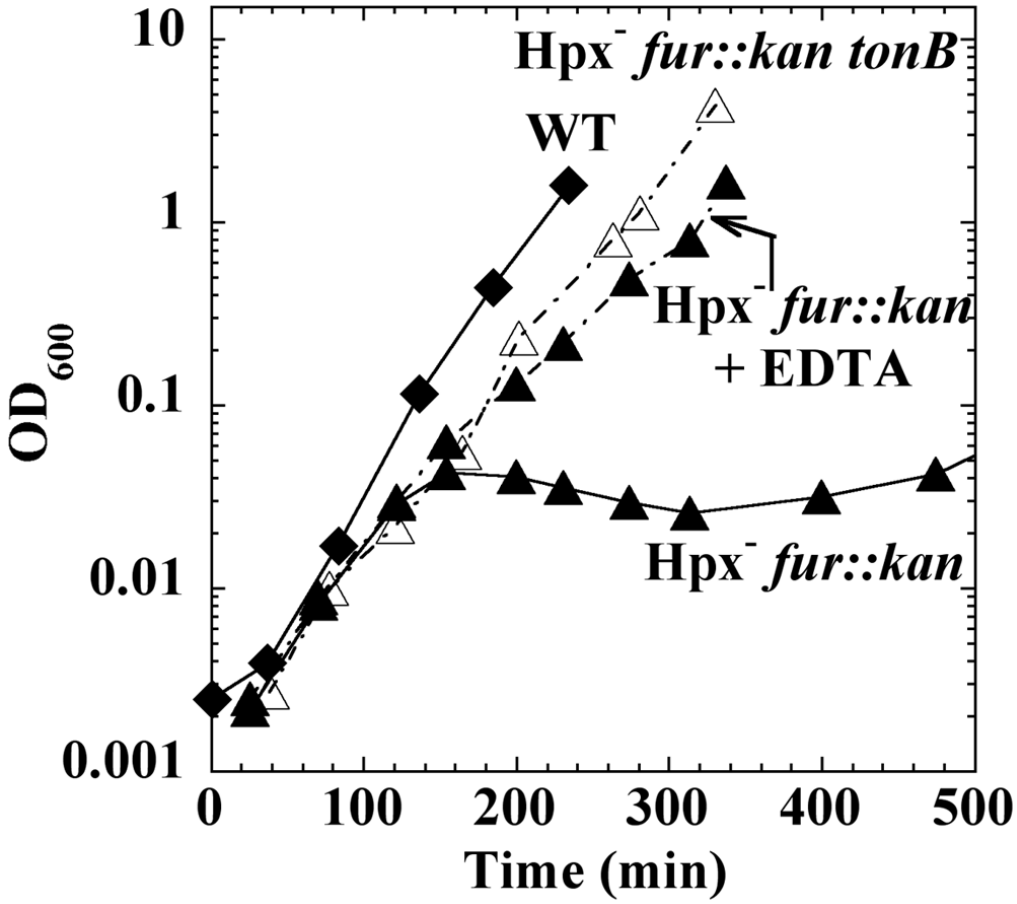

Figure 6. Role of iron import systems in the growth defects of *fur* mutants

Cultures were grown in aerobic LB medium. Hpx⁻ *fur::kan* (KCI323, filled triangles), Hpx⁻ *fur::kan tonB* (AW510, open triangles), and wild-type (MG1655, diamonds) strains are shown. Where indicated, EDTA was included in the medium.

Table 1

Strain	Genotype	Reference
BW381	As GC4468 plus *soxS::*Tn*10*	(Tsaneva and Weiss, 1990)
SMV37	As LC106 plus *soxS::*Tn*10*	P1(BW381) × LC106
LC106	Δ*ahpCF' kan::'ahpF* Δ (*katG17*::Tn*10*)1 Δ(*katE12*::Tn*10*)1	(Seaver and Imlay, 2004)
GI69	*zdg-299::*Tn*10*	(Grogan and Cronan, 1984)
JI132	(*sodA::Mud* PR13)25 (*sodB::kan)1-Δ2*	Lab stock
KK183	As JI132 plus *zdg-299::*Tn*10*	P1(G169) × JI132
SMV29	As LC106 plus (*sodB::kan)1-Δ2*	P1(KK183) × LC106
SMV32	As MG1655 plus (*sodB::kan) 1-Δ2*	P1(KK183) × MG1655
SMV31	As LC106 plus (*sodA::Mud* PR13)25	P1(KK183) × LC106
KCI420	As MG1655 plus (*sodA::Mud* PR13)25	P1(PN134) × MG1655
RZ8457	As MG1655, As MG1655, Δ*fnr (Bsm-Mlu)::Ω(Sp^R), zcj::*Tn*10*	P. Kiley
SMV33	As MG1655 plus Δ*fnr (Bsm-Mlu)::Ω(Sp^R), zcj::*Tn*10*	P1(RZ8457) × MG1655
SMV34	As LC106 plus Δ*fnr (Bsm-Mlu))::Ω(Sp^R), zcj::*Tn*10*	P1(RZ8457) × LC106
EM1456	As MG1655 *lacx74* Δ *ara714 ryhB::cam* with pNM12	E. Masse
SMV36	As LC106 plus *rhyB::cam*	P1(EM1456) × LC106
KK1445	*zhh-5::*Tn*10*	*E. coli* Genetic Stock Center
SMV380	As EM1456 plus *zhh-5::*Tn*10*	P1(KK1445) × EM1456
SMV40	As SMV31 plus *ryhB::cam~zhh-5::*Tn*10*	P1(SMV380) × SMV31
CGSC6391	*Hfr valS uxuBA zbf-507::*Tn*10 (near 15.7') trp-49 (Am), lacZ125 (Am), relA1, spot1, l-*	*E. coli* Genetic stock center
BW25113	Plus pKD46	(Datsenko and Wanner, 2000)
SP81	As BW25113 plus *zbf-507::*Tn*10*	P1(CGSC6391) × BW25113
SP82	As SP81 plus *fur::cam^{NI}*	This work
SP84	As BW25113 plus *fur^{NI}~zbf-507::*Tn*10 (asnB-507::Tn10)*	This work
SMV49	As MG1655 plus *fur::cam^{NI}~*Tn*10*	P1(SP82) × MG1655
SMV50	As LC106 plus *fur::cam^{NI}~*Tn*10*	P1(SP82) × LC106
SMV52	As MG6155 plus *fur::cam^{NI}*	P1(SP82) × MG1655
SMV53	As LC106 plus *fur::cam^{NI}*	P1(SP82) × LC106
SMV55	As MG1655 plus *fur^{NI}~zbf-507::*Tn*10*	P1(SP84) × MG1655
SMV57	As LC106 plus *fur^{NI}~zbf-507::*Tn*10*	P1(SP84) × LC106
BN407	As AB1157 pColV*::lacZ (iucC::lacZ)*	(Bagg and Neilands, 1985)
SMV59	As SMV57 plus pcolV*::lacZ(iucC::lacZ)*	BN407 × SMV57
MS100	As MG1655 plus pcolV*::lacZ(iucC::lacZ)*	BN407 × MG1655
MS102	As LC106 plus pcolV*::lacZ(iucC::lacZ)*	BN407 × LC106
KK204	As AB1157 plus *fur::kan*	Lab stock
KK210	*fur::kan~ zbf-507::*Tn*10.*	Lab stock
KCI323	As LC106 plus *fur::kan~zbf-507::*Tn*10*	P1(KK210) × LC106
AW25113	As BW25113 plus *tonB::cat*	This work

Strain	Genotype	Reference
AW510	As KCI323 plus *tonB::cat*	P1(AW25113) × KCI323