The YaaA Protein of the *Escherichia coli* OxyR Regulon Lessens Hydrogen Peroxide Toxicity by Diminishing the Amount of Intracellular Unincorporated Iron†‡

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Hydrogen peroxide (H$_2$O$_2$) is commonly formed in microbial habitats by either chemical oxidation processes or host defense responses. H$_2$O$_2$ can penetrate membranes and damage key intracellular biomolecules, including DNA and iron-dependent enzymes. Bacteria defend themselves against this H$_2$O$_2$ by inducing a regulon that engages multiple defensive strategies. A previous microarray study suggested that yaaA, an uncharacterized gene found in many bacteria, was induced by H$_2$O$_2$ in *Escherichia coli* as part of its OxyR regulon. Here we confirm that yaaA is a key element of the stress response to H$_2$O$_2$. In a catalase/peroxidase-deficient (Hpx$^-$) background, yaaA deletion mutants grew poorly, filamented extensively, and lost substantial viability when they were cultured in aerobic LB medium. The results from a thyA forward mutagenesis assay and the growth defect of the yaaA deletion in a recombination-deficient (recA56) background indicated that yaaA mutants accumulated high levels of DNA damage. The growth defect of yaaA mutants could be suppressed by either the addition of iron chelators or mutations that slowed iron import, indicating that the DNA damage was caused by the Fenton reaction. Spin-trapping experiments confirmed that Hpx$^-$ yaaA cells had a higher hydroxyl radical (HO$^-$) level. Electron paramagnetic resonance spectroscopy analysis showed that the proximate cause was an unusually high level of intracellular unincorporated iron. These results demonstrate that during periods of H$_2$O$_2$ stress the induction of YaaA is a critical device to suppress intracellular iron levels; it thereby attenuates the Fenton reaction and the DNA damage that would otherwise result. The molecular mechanism of YaaA action remains unknown.

The first living organisms appeared on an Earth that was anaerobic and that remained so for another 2 billion years (5). By that point, however, photosynthetic processes had generated enough oxygen to titrate the reduced iron and sulfur species in the ancient seas, and over the next billion years the continued production of oxygen led to its accumulation in the atmosphere until concentrations reached present-day levels. During this period, microbial organisms evolved mechanisms to exploit the metabolic potential of oxygen—but they also had to devise strategies to defray the hazards it posed. Molecular oxygen is a triplet species that reacts rapidly with organic radicals; therefore, once aerobic environments appeared, ancient free-radical-based catalytic mechanisms were either dispensed with or were sequestered in the interiors of enzymes, where oxygen could not penetrate.

A second threat consisted of superoxide and hydrogen peroxide, partially reduced forms of oxygen that are more potent oxidants than is oxygen itself. These species are routinely generated within aerobic cells, apparently when molecular oxygen steals electrons from the reduced flavins or quinones of redox enzymes (27, 28, 36). Their potential for toxicity is implied by the high titers of scavenging enzymes—superoxide dismutases and reductases, and catalases and peroxidases—that are found in virtually all living cells. Mutant bacteria that lack these enzymes do not exhibit phenotypic defects as long as they remain in anaerobic habitats, but upon exposure to oxygen they grow poorly, suffer high rates of mutagenesis, or die (7, 43). Biochemical and physiological studies of such mutants have allowed the identification of some of the targets with which superoxide and H$_2$O$_2$ react most avidly. Both superoxide and hydrogen peroxide oxidize the exposed iron-sulfur clusters of aconitase class dehydratases, precipitating their degradation and the loss of enzyme activity (12, 23, 30). Hydrogen peroxide also reacts directly with ferrous iron, and this reaction inactivates enzymes that use mononuclear ferrous iron cofactors (45a). A similar reaction on the surface of DNA produces hydroxyl radicals, and their subsequent oxidation of nucleic acid bases or ribose moieties can cause mutagenesis or cell death (20, 40).

In wild-type *Escherichia coli*, the best-studied system, the levels of scavenging enzymes are sufficient to drive the steady-state concentration of superoxide below nanomolar levels and the H$_2$O$_2$ concentration to about 10$^{-8}$ M (21, 44). These concentrations are low enough to avoid any fitness defect, as higher expression of scavenging enzymes provides no further stimulus to aerobic growth. Thus, the basal defenses of bacteria appear to be calibrated to avoid problems from endogenous oxidants.

Nevertheless, bacteria may be critically damaged by H$_2$O$_2$ when it enters the cell at high rates from the external environment. Hydrogen peroxide is produced when oxygen reacts with metals and thiols, a phenomenon that occurs in the interfaces between aerobic and anaerobic habitats. Because of its toxicity,
H₂O₂ is also used as a weapon in a variety of natural antimicrobial systems: plants, amoeboae, and mammals all elaborate an NADPH oxidase to kill invading bacteria (4, 13, 35), and both plants and microbes secrete redox-cycling phenazines and quinones to suppress the growth of their competitors (22, 48). Hydrogen peroxide penetrates membranes at a rate similar to that of water (44), so when bacteria enter an environment that contains H₂O₂, they must activate defensive responses. The first of these to be found, the OxyR system, is triggered when peroxide oxidizes an activated thiolate residue to a sulfenic acid on the OxyR transcription factor (31, 55). The activated OxyR protein then directly stimulates transcription of genes involved in defense. Microarray studies performed in E. coli have suggested that at least two dozen genes lie within the OxyR regulon (57).

These genes presumably signify a list of cellular strategies to fend off the toxic actions of H₂O₂. Not surprisingly, they include katG and ahpCF, which encode the primary catalase and peroxidase in the cell (43). Dps is a ferritin-like protein that sequesters unincorporated iron and thereby reduces its role in damaging DNA (14, 19, 40, 54); the Fur repressor is also induced (56), and it diminishes iron import with the same end effect (49). Hydrogen peroxide directly poisons the housekeeping Isc iron-sulfur cluster assembly system (24), and the induction of the Suf operon provides an H₂O₂-resistant replacement (32, 38). Suf induction is therefore also critical for the repair of damaged iron-sulfur clusters. The induced synthesis of a manganese importer, MntH, enables manganese to replace iron damaged iron-sulfur clusters. The induced synthesis of a manganese importer, MntH, enables manganese to replace iron

In the absence of iron, Dps is induced (32, 38). Dps is a ferritin-like protein that sequesters unincorporated iron and thereby reduces its role in damaging DNA (14, 19, 40, 54); the Fur repressor is also induced (56), and it diminishes iron import with the same end effect (49). Hydrogen peroxide directly poisons the housekeeping Isc iron-sulfur cluster assembly system (24), and the induction of the Suf operon provides an H₂O₂-resistant replacement (32, 38). Suf induction is therefore also critical for the repair of damaged iron-sulfur clusters. The induced synthesis of a manganese importer, MntH, enables manganese to replace iron.

The significance of these adaptations is evident in E. coli mutants that lack both catalase and peroxidase (katG katE ahpCF, or Hpx⁻). These strains accumulate ca. 1 μM H₂O₂, which is slightly above the dose that triggers expression of the OxyR regulon (3, 44). Thus, whereas dps or mntH single mutants can tolerate oxygen, Hpx⁻ dps and Hpx⁻ mntH mutants are viable only in anaerobic medium (2, 40).

The microarray data (57) indicated that the OxyR regulon also includes several genes whose roles are not obvious. One of these, yaaA, is predicted to encode a 29.6-kDa cytoplasmic protein that belongs to a protein family domain of unknown function (DUF328). To explore the role of YaaA during H₂O₂ stress, we have examined the phenotype of a gene deletion in E. coli (57).

\[ \text{YaaA AND HYDROGEN PEROXIDE IN } E. \text{ COLI} \]

\[ \text{Strain constructions.} \]

The strains used in this study are listed in Table 1. Deletions were constructed by the Red recombination deletion method (10) and were transferred to other strains by P1 transduction (37). The antibiotic resistance cassettes were subsequently removed by FLP-mediated excision (10). Gene fusions between the yaaA upstream region (bp −290 to −1) and lacZ were created by standard methods and integrated into the lambda phage attachment site (18), preserving the wild-type yaaA locus at its native chromosomal locus.

\[ \text{Plasmid construction.} \]

The yaaA coding sequence was amplified by PCR from E. coli MG1655 genomic DNA using the forward primer 5'-TTTCAAGATTC TAAAATTTCTGCAAGGACGTG-3' and the reverse primer 5'-GATTCCCTA GACAAATTTAACGCTGCTGTA-3'. These oligonucleotide primers were designed to engineer EcoRI and XbaI sites at the 5' and 3' ends of the gene, respectively. The PCR products were purified, digested with EcoRI and XbaI, and cloned into the pWKS30 vector (50), so that yaaA expression was controlled by the lac promoter. The correctness of the plasmid construct was confirmed by sequencing.

\[ \text{Bacterial growth.} \]

Complex growth medium was LB (10 g Bacto tryptone, 5 g yeast extract, and 10 g NaCl per liter). Defined glucose/amino acids medium contained minimal A salts (37), 0.2% glucose, 0.2% Casamino Acids, 0.5 mM tryptophan, 0.02% MgSO₄, 7H₂O, and 5 μg/ml thiamine. When antibiotic selection was required, media were supplemented with 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, 30 μg/ml kanamycin, 100 μg/ml spectinomycin, or 12 μg/ml tetracycline.

Experimental protocols were designed to ensure that measurements were performed upon exponentially growing cells. For growth studies, anaerobic overnight cultures were diluted into anaerobic medium and grown for four to five generations to early log phase (optical density at 600 nm [OD₆00] of 0.15 to 0.20). The cultures were then diluted into aerobic medium of the same composition to an OD₆00 of 0.0005 to 0.010. All cultures were grown at 37°C. Aerobic LB medium was made 1 day prior to culturing and stored in the dark to avoid the photochemical generation of H₂O₂. Aerobic defined medium was prepared immediately prior to use. Anaerobic medium was transferred immediately after autoventing to an anaerobic chamber (Coy Laboratory Products), where it was stored under an atmosphere of 5% CO₂–10% H₂–85% N₂ for at least 1 day prior to use. When used, DFO was made fresh and added into the medium at a final concentration of 2 mM before inoculation.

\[ \text{Cell viability.} \]

To determine the cell viability, cell samples were collected at designated time points and mixed with 250 U/ml catalase (final concentration) to scavenge residual hydrogen peroxide. Samples were then serially diluted, mixed with top agar, and spread onto the surface of anaerobic plates. Colonies were allowed to grow anaerobically for 24 to 48 h before counting.

\[ \text{DIC microscopy.} \]

Cultures were grown in anaerobic LB medium to exponential phase before dilution into aerobic LB medium to an OD₆00 of 0.0005. They were then grown for 5 h. Images were captured by differential interference contrast (DIC) microscopy (17).

\[ \text{thyA forward mutagenesis assay.} \]

In order to determine the frequency of mutagenesis, Thy⁻ mutants were selected using TMP (37). Strains were transferred from anaerobic LB to aerobic LB as described above. At intervals aliquots were removed, mixed with top agar to stop oxidative stress, and serially diluted into minimal glucose/amino acids medium at room temperature three times. The cells were then cultured in an anaerobic chamber and diluted into minimal A salts. The number of viable cells was determined by dilution and plating in 2 ml F-top agar containing 1 mg/ml thymidine on defined glucose/amino acids plates. Thy⁻ mutants were selected by including 0.2 mg/ml TMP in the F-top agar, and CFU were determined after anaerobic incubation at 37°C for 48 h. For each experiment, a subset of the trimethoprim-resistant mutants was tested and confirmed to be Thy⁻ by examining their growth on minimal glucose/amino acids plates with and without thymidine supplementation.

\[ \text{H}_2\text{O}_2 \text{ killing assay.} \]

Cells were grown in minimal A glucose medium containing 0.5 mM tryptophan, phenylalanine, and tyrosine to an OD₆00 of 0.15 as described above. The cultures were then diluted to an OD₆00 of 0.025 in the same medium and incubated for 5 min with either 3.3 mM potassium cyanide (53) or 0.5 mM cystine (39). Hydrogen peroxide (2.5 mM) was then added. At the indicated time points aliquots were removed, and 2,500 U/ml catalase (final concentration) was added in order to end the hydrogen peroxide stress. After serial dilution, samples were mixed with top agar and spread on minimal A defined medium plates. CFU were determined after anaerobic incubation at 37°C for 48 h.

\[ \text{EPR spin trapping of hydroxyl radicals.} \]

Cells were cultured in aerobic LB as described above. The cells were then harvested at an OD₆00 of 0.2, when the growth of Hpx⁻ yaaA cells slowed down. The cells were washed twice with 25 ml of ice-cold Chelex-treated HBSS and resuspended in 0.5 ml of ice-cold HBSS. The cell suspension was added to a room temperature mixture (total volume, 1 ml) containing 100 mM DTPA, 10 mM 4-POBN, 170 mM ethanol, 1 mM H₂O₂.
and HBSS, and the POBN hydroxyl radical adducts were quantified by electron paramagnetic resonance spectroscopy (EPR) as described previously (33). The final cell densities were equivalent in all samples (OD_{600} of ~20).

**EPR measurement of intracellular unincorporated iron.** The cells were cultured in 600 to 1,000 ml of aerobic LB medium as described above. The cell pellets were washed and resuspended at a 100-fold-higher cell density in LB medium containing 10 mM DTPA (pH 7.0) and 20 mM DFO (pH 8.0). The suspension was incubated at 37°C aerobically for 15 min. The purpose of the cell-impermeable chelator DTPA was to block further iron import, while the cell-penetrating chelator DFO facilitated oxidation of unincorporated ferrous iron to EPR-detectable ferric iron, as described previously (52). The cell pellet was washed twice with 5 ml of ice-cold 20 mM Tris-HCl–30% glycerol (pH 7.4). The EPR analysis was performed as described previously (33). The following primers were used to analyze expression of the intracellular unincorporated iron: 5′- ACTACGGGTTAAGGACATCACCTT-3′ and the housekeeping gene gapA forward, 5′-GCTGTGACGTAGACTACCCCTT-3′. Thermal cycling was performed for 35 cycles in three steps: 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min.

**Visualization of protein carbonylation.** Cells were cultured in aerobic LB as described above. Hpx-yaaA cells were harvested by centrifugation at the beginning (OD_{600} 0.2) and in the middle of the growth lag (OD_{600} ~0.25). Other strains were collected at an OD_{600} of ~0.2. Cell pellets were washed with ice-cold 50 mM potassium phosphate buffer, pH 7.0, and resuspended in the same buffer with 5 mM DTPA, which prevented further protein oxidation in cell extracts. After lysis using a French press, cell debris was removed by centrifugation, and protein concentrations in the cell extracts were determined using the Bradford assay. All samples were then diluted to the same protein concentration (6 mg/ml). Protein carbonyl groups were derivatized with DNP, and the adducts were visualized by Western blotting (2).

**Rapid amplification of cDNA ends (5′-RACE).** MG1655 cells and Hpx-yaaA cells were cultured in LB as described above and harvested at an OD_{600} of ~0.2. RNA was extracted by the hot phenol method. Contaminating DNA was removed by DNase I digestion (Turbo DNA-free kit; Ambion), and the DNA-free RNA samples were converted to cDNA according to the manufacturer’s instructions (SuperScript II reverse transcriptase; Invitrogen). The 5′ end of yaaA transcripts was determined by using a FirstChoice RLM-RACE Kit (Applied Biosystems). The PCR primers used were yaaA inner 5′- AGCCGGTGTAGACATCACCTTT-3′ and gapA inner 5′- AGCCGGTGTAGACATCACCTTT-3′. Thermal cycling was performed for 35 cycles in three steps: 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min.

**Real-time PCR.** Cells were cultured in LB as described above. When cultures achieved an OD_{600} of ~0.2, they were treated with H_{2}O_{2} (0.025, 1, and 2 mM) for 10 min at room temperature. mRNA was harvested and converted to cDNA as described above. The following primers were used to analyze expression of yaaA and the housekeeping gene gapA: yaaA forward, 5′- CGCCGGTACGTAGAACAAT TCCACGACG-3′; yaaA reverse, 5′- AAGCCGGTGTAGACATCACCCCTT-3′; gapA forward, 5′- GCTGGTGGTCACGGAGATGACG-3′; gapA reverse, 5′- AAGCCGGTGGTCACGGAGATGACG-3′. The 25-μl real-time PCR mixture contained 1 ng of cDNA, 12.5 μl of SYBR Green supermix (Bio-Rad), and 1.6 μM each primer. Thermal cycling was performed in a Mastercycler ep realplex.
RESULTS

The gene yaaA is induced by OxyR. The gene yaaA is predicted to encode a 29.6-kDa conserved cytoplasmic protein that belongs to a family domain of unknown function, DUF328 (11). YaaA does not possess amino acid similarity to proteins that have defined functions, and it does not have any recognizable motifs. DNA microarray data from Zheng et al. showed that yaaA was induced in an OxyR-dependent manner when 1 mM H₂O₂ was applied (57). Our analysis of the DNA sequence upstream of yaaA revealed a potential OxyR binding site approximately 74 bp upstream of the yaaA start codon (Fig. 1). To verify these observations, we constructed a yaaA-lacZ transcriptional fusion and measured the expression of yaaA in different strains.

When transferred from anaerobic to aerobic conditions, the expression of yaaA was not induced in wild-type cells (E. coli MG1655). However, under the same conditions its expression was induced 10-fold in a catalase/peroxidase-deficient strain MG1655. However, under the same conditions its expression was induced 10-fold in a catalase/peroxidase-deficient strain MG1655. When wild-type cells were transformed with the plasmid pGSOS8, which contains a mutated oxyR allele (A233V) that constitutively overexpresses OxyR-regulated genes; aph (AL145), NADH peroxidase mutant; Hpx (AL145), catalase/peroxidase-deficient mutant. (B) Anaerobic exponential-phase cultures were harvested at an OD₆₀₀ of ~0.2 (~O₂) or were aerated for 30 min before collection (~O₂). The OD₆₀₀ of the Hpx oxyR⁻ (AL145) and Hpx oxyR mutant (AL344) strains increased from ~0.2 to ~0.35 during the period of aeration. In this and other figures, the error bars represent the standard errors of the means.

When transferred from anaerobic to aerobic conditions, the expression of yaaA was not induced in wild-type cells (E. coli MG1655). However, under the same conditions its expression was induced 10-fold in a catalase/peroxidase-deficient strain MG1655. When wild-type cells were transformed with the plasmid pGSOS8, which contains a mutated oxyR allele (A233V) that constitutively overexpresses OxyR-regulated genes even in the absence of H₂O₂ stress (29), the expression of yaaA was induced under both anaerobic (data not shown) and aerobic conditions (Fig. 2A). These results confirmed that yaaA is part of the OxyR regulon.

Interestingly, in aph-deficient (catalase-proficient) mutants that experience minor H₂O₂ stress (44), the expression of yaaA was at the basal level during aerobic culture (Fig. 2A), even though katG, another member of the OxyR regulon, was strongly induced (data not shown). The result suggests either that the yaaA promoter region has a lesser affinity for oxidized OxyR, or that a modifying effect is imposed by another regulator that is affected by more severe H₂O₂ stress.

Hpx⁻ yaaA cells show a growth defect in LB during H₂O₂ stress. Since yaaA was induced in Hpx⁻ strains during aerobic culture, we constructed a yaaA deletion in the catalase/peroxidase-deficient background (Hpx⁻ yaaA). The Hpx⁻ yaaA cells grew normally under anaerobic conditions, but they stopped growing about five generations after shifting to aerobic conditions, lagging for several hours before recovering (Fig. 3A).

The aerobically grown Hpx⁻ yaaA cultures filamented profusely (Fig. 4). Both anaerobic cultures and aerobic Hpx⁻ yaaA cultures appeared as normal unit-sized cells (data not shown), confirming that H₂O₂ was needed to create this phenotype. Filamentation is a hallmark of difficulty in DNA replication, and it commonly results when chemical stress damages DNA but does not substantially disrupt protein or membrane biogenesis.

A chromosomal insertion of the yaaA⁺ allele at the λ phage attachment site suppressed the growth defect and the filamentation of Hpx⁻ yaaA cells (data not shown). Deletion of yaaJ, which terminates at 70 bp upstream of yaaA, in the Hpx⁻ strain, does not result in a growth defect during H₂O₂ stress (data not shown). These results indicated that the growth defect of the Hpx⁻ yaaA mutant was due to the loss of YaaA function and that YaaA is involved in the cellular response to H₂O₂ stress.

Hpx⁻ yaaA cells lose viability during H₂O₂ stress. The viability of Hpx⁻ yaaA strains and Hpx⁻ parent strains were monitored upon aeration (Fig. 5). Samples were harvested at different time points, and CFU were determined by plating in LB top agar. After a brief lag, the number of viable cells increased steadily in the Hpx⁻ parent strain. In contrast, the viable cell count of the Hpx⁻ yaaA strain did not change during the first 2 h of aerobic culture, confirming that the increase in the OD₆₀₀ was due to filamentation rather than cell division.
Approximately 3 h after aeration, concomitant with the onset of the growth lag, the viability of the Hpx\(^{-}\) yaaA mutant decreased by an order of magnitude.

Ultimately, the number of viable cells began to increase again, albeit at a slower pace than in the yaaA\(^{-}\) parent. This recovery was not due to suppressor mutations, since the isolated outgrowers reproduced the same growth pattern when the experiment was repeated. Some of the outgrowth depended upon conditioning of the medium, since a lag resumed when the outgrowing cells were centrifuged and then diluted into fresh aerobic medium. The Hpx\(^{-}\)/H11002 strains can gradually degrade H\(_2\)O\(_2\) that is found in LB medium, because the H\(_2\)O\(_2\) is a weak substrate for the cytochrome oxidases of the respiratory chain (A. L. Woodmansee and J. A. Imlay, unpublished data); a similar recovery has been observed in Hpx\(^{-}\) strains that lack fur or dps, two other genes that are critical during H\(_2\)O\(_2\) stress (40, 49).

The expression of yaaA was also strongly induced when Hpx\(^{-}\) strains were aerated in defined glucose medium (data not shown). However, in contrast to the results in LB broth, the Hpx\(^{-}\) yaaA mutant did not exhibit any phenotype in defined glucose medium (data not shown). The growth defect re-emerged when yeast extract was added to the defined medium (see Discussion).

**yaaA mutants show higher levels of DNA damage and polypeptide damage during H\(_2\)O\(_2\) stress.** Previous work has shown that H\(_2\)O\(_2\) directly inactivates enzymes that employ solvent-exposed iron atoms either in iron-sulfur clusters or as mononuclear cofactors (23, 45a). Further, H\(_2\)O\(_2\) generates hydroxyl radicals when it reacts with unincorporated intracellular ferrous iron, and these radicals then covalently damage both DNA and proteins (2, 20, 40). We sought to identify the cellular injury that arrests the growth of Hpx\(^{-}\) yaaA mutants, in order to gain insight into the function of YaaA.

The filamentation and cell death of Hpx\(^{-}\) yaaA strains suggested that DNA might be damaged. Indeed, a thyA forward mutagenesis assay revealed that the mutation frequency of aerobically grown Hpx\(^{-}\) yaaA cells was 4- to 8-fold higher than that of the Hpx\(^{-}\) strain (Fig. 6). The effect depended upon oxidative stress, since the yaaA mutant in the wild-type (scavenger-proficient) background exhibited a mutation frequency similar to that of the parent strain. The yaaA mutation did not

**FIG. 3.** The Hpx\(^{-}\) yaaA strain exhibits a growth defect in aerobic LB medium. Exponential-phase cultures in anaerobic LB medium were diluted into aerobic LB medium at time zero, and the optical density was monitored. (A) Wild-type cells (MG1655), black squares; ΔyaaA (AL245), gray triangles. The symbols for Hpx\(^{-}\) (catalase/peroxidase-deficient strain, LC106) and Hpx\(^{-}\) yaaA (SB53) are defined in the figure. (B) Symbols (in addition to those defined for panel A): open circles, Hpx\(^{-}\) yaaA cells with 2 mM DFO in the medium; stars, Hpx\(^{-}\) tonB yaaA (AL179).

**FIG. 4.** Hpx\(^{-}\) yaaA cells exhibit a severe filamentous phenotype. Exponentially growing cultures in anaerobic LB medium were diluted into aerobic LB medium and grown for 5 h. Images were captured by DIC microscopy using a 40\(\times\) oil immersion objective.

**FIG. 5.** The growth defect of the Hpx\(^{-}\) yaaA mutant occurred concomitantly with cell death. Exponentially growing cultures in anaerobic LB medium were diluted into aerobic LB medium at time zero. (A) Growth rates, based on the optical density. (B) Viable cell counts for Hpx\(^{-}\) (LC106) and Hpx\(^{-}\) yaaA (SB53).
sensitize cells to UV radiation, an alternative source of DNA damage (data not shown).

In a scavenger-proficient background, the yaaA mutation did create a growth defect in recA56 strains (Fig. 7A and B), which are defective in the repair of oxidative DNA lesions (1). This defect was apparent, once again, only in LB medium and only under aerobic conditions. The defect was blocked when catalase was added to the medium (Fig. 7C), which implied that the damage was due to the trace H$_2$O$_2$ that is present in aerobic LB medium. A similar result was obtained with polA1 mutants (see Fig. S1 in the supplemental material). The defect is transient, due to the decomposition of H$_2$O$_2$ by cellular enzymes. These data indicate that (i) YaaA has a role independent of the DNA recombination pathway or the SOS response, which are already absent from recA56 mutants, and (ii) even very low levels of H$_2$O$_2$ can cause debilitating DNA damage in the yaaA mutant. The data, however, do not resolve whether YaaA has a role in preventing DNA damage or in repairing it.

During H$_2$O$_2$ stress, the hydroxyl radicals that are produced by the Fenton reaction can damage both DNA and polypeptides. The latter injury can be tracked by the formation of carbonyl adducts, which arise when hydroxyl radicals oxidize some amino acid side chains. An enzyme-linked immunosorbent assay-based carbonylation assay did not detect any difference in the extent of protein carbonylation in Hpx$^-$/H11002yaaA and Hpx$^+$/H11002yaaA/H11001 mutants when they were grown in defined glucose medium (data not shown). However, when they were cultured in aerobic LB medium, the Hpx$^-$/H11002yaaA cells gradually accumulated higher levels of protein carbonyls than did the Hpx$^+$ parent strain. These modifications were apparent at the later time point, which coincides with the growth lag (Fig. 8). While the most straightforward interpretation is that the dysfunction of YaaA directly causes the higher carbonylation, it remains possible that the carbonylation is an indirect consequence of the growth defect.

We also evaluated whether YaaA has a role in defending mononuclear iron-containing enzymes or solvent-exposed Fe-S clusters against H$_2$O$_2$ stress. The activities of ribulose-5-phosphate epimerase and isopropylmalate isomerase, respectively, were compared in Hpx$^-$/H11002 and Hpx$^-$/H11002yaaA mutants. Both enzymes exhibited a reduction in activities relative to scavenger-proficient strains, but the yaaA allele had no further impact (data not shown).

The Fenton reaction causes injuries in yaaA mutants during H$_2$O$_2$ stress. The preceding results suggested that yaaA might suppress cellular injuries that arise from the Fenton reaction. Consistent with this idea, the growth defect of Hpx$^-$ yaaA mutants was suppressed by maneuvers that diminished the amount of intracellular iron. The exogenous addition of DFO, a cell-permeable iron chelator that blocks Fenton chemistry (20), eliminated the growth lag (Fig. 3B). A similar effect was obtained by a tonB mutation, which prevents iron import by the
siderophore and ferric-citrate systems (51) (Fig. 3B). Furthermore, DFO also eliminated the growth defect of recA yaaA mutants (Fig. ?D). These results confirm that the key injury in the yaaA mutant during H2O2 stress is due to the Fenton reaction.

YaaA protects cells by attenuating the Fenton reaction. In principle, YaaA might protect cells either by attenuating the Fenton reaction and reducing HO\textsuperscript{•} formation or by helping cells to tolerate the damage that it creates. In order to distinguish between these possibilities, we employed EPR to evaluate the rates of HO\textsuperscript{•} formation.

Intracellular HO\textsuperscript{•} was trapped by ethanol/POBN and detected by room temperature EPR. Wild-type cells exhibited a vanishingly small signal (data not shown), whereas the level of hydroxyl radical formation was substantial in Hpx\textsuperscript{−} mutants, as reported previously (40). In the early period after aeration, the Hpx\textsuperscript{−} yaaA cells exhibited a signal similar to that of Hpx\textsuperscript{−} cells (data not shown). However, the signal continually increased in the Hpx\textsuperscript{−} yaaA cells, ultimately becoming about twice that of the Hpx\textsuperscript{−} strain (Fig. 9). Pretreatment with 2 mM dipiridyl (an iron chelator) for 5 min eradicated the EPR signal (data not shown), confirming that it represented hydroxyl radicals produced by the Fenton reaction. These results indicated that YaaA protects cells by attenuating the Fenton reaction.

YaaA does not affect H2O2 levels. Continuous hydroxyl radical formation results when the cellular pool of unincorporated iron cycles through two consecutive reactions, the Fenton reaction (termed reaction 1 here; Fe\textsuperscript{2+} + H2O2 \rightarrow Fe\textsuperscript{3+} + OH\textsuperscript{−} + HO\textsuperscript{•}) followed by the regeneration of ferrous iron mediated by cellular reductants (reaction 2; Fe\textsuperscript{3+} + red \rightarrow Fe\textsuperscript{2+} + ox).

Both intracellular cysteine and reduced flavins have been shown to serve as the predominant reductants in reaction 2, depending upon the growth conditions (39, 53). To minimize oxidative injuries, cells must control the levels of H2O2, unincorporated iron, and cysteine/flavin. The spin-trapping data suggested that YaaA suppresses the level of one of these reactants.

The rate of intracellular H2O2 formation depends upon the titers of autoxidizable enzymes in the cell, and in Hpx\textsuperscript{−} strains the steady-state level of H2O2 also depends upon minor scavenging mechanisms. We sought to test whether YaaA might affect either of these processes. In Hpx\textsuperscript{−} strains the H2O2 concentration rapidly equilibrates between the cytoplasm and the external medium; therefore, cocultured Hpx\textsuperscript{−} strains experience equivalent intracellular concentrations of H2O2, and a strain that efficiently scavenges H2O2 can suppress the growth defects that would otherwise arise in a cocultured strain that cannot (44). To this end, we cocultured Hpx\textsuperscript{−} cells and Hpx\textsuperscript{−} yaaA cells together in LB medium under aerobic conditions and then analyzed the viability at designated time intervals for the two individual strains, which were distinguished upon plating by using drug resistance markers. In mixed culture the Hpx\textsuperscript{−} yaaA strain continued to exhibit its full aerobic viability defect, while the Hpx\textsuperscript{−} strain did not. The result indicates that YaaA does not alter the intracellular levels of H2O2 (data not shown).

The rate of cellular H2O2 formation was also directly measured as described in Materials and Methods. These experiments were performed in defined media, as components in LB interfere with H2O2 measurements. Under these conditions, the rate of H2O2 accumulation in Hpx\textsuperscript{−} cells was 11 ± 1 nM/min (mean ± standard error of the mean), versus 11 ± 0.3 nM/min in Hpx\textsuperscript{−} yaaA cells, which further indicates that YaaA does not affect the rate of H2O2 accumulation.

YaaA does not inhibit ferric iron reduction. FADH2 and cysteine can each reduce ferric iron and drive the Fenton reaction forward in vivo. Reduced free flavin concentrations are elevated by respiratory blocks, through the action of an NADH-dependent flavin reductase (Fre), and under these conditions H2O2 can create hydroxyl radicals and DNA lesions at a very high rate (53). However, in defined medium neither deletion nor induction of the yaaA allele affected the rate at which cyanide/H2O2 killed cells (data not shown). The same was true when cells were shifted from sulfate to cystine medium (data not shown), a protocol that elevates intracellular cysteine levels and similarly amplifies H2O2 sensitivity (39). These results suggest that YaaA is unlikely to attenuate the Fenton reaction by inhibiting the rate of ferric iron reduction (reaction 2).

Hpx\textsuperscript{−} yaaA mutants have higher unincorporated iron levels than the Hpx\textsuperscript{−} parent strain. The rate of oxidative DNA damage is directly determined by the amount of unincorporated iron inside the cell (26, 46). To learn whether YaaA controls iron levels, we measured intracellular unincorporated iron by whole-cell EPR. Samples were cultured aerobically in LB and collected at an OD\textsubscript{600} of 0.1, 0.2, and 0.3; these time points represented Hpx\textsuperscript{−} yaaA cells prior to the growth lag, at its onset, and at its midpoint. The data showed that deletion of yaaA in unstressed Hpx\textsuperscript{−} cells had no effect on the iron levels of approximately 65 μM (Fig. 10A). The level of unincorporated iron in H2O2-stressed Hpx\textsuperscript{−} strains was substantially higher (150 μM), an effect that may reflect the oxidative degradation of iron-sulfur clusters. The key observation, however, was that the Hpx\textsuperscript{−} yaaA cells consistently accumulated 2-fold-higher levels of unincorporated iron (300 μM) by the begin-
Consistent with the growth curve results, the EPR data showed that the iron returned to Hpx\textsuperscript{-} levels when yaaA was expressed in trans on a plasmid. This effect matches the 2-fold effect of yaaA upon hydroxyl radical formation. The iron returned to Hpx\textsuperscript{-} levels when yaaA was expressed in trans on a plasmid (Fig. 10B).

Hpx\textsuperscript{-} yaaA cells do not show a growth defect compared to Hpx\textsuperscript{-} parent strains in defined medium during aerobic culture. Consistent with the growth curve results, the EPR data showed that the level of intracellular free iron in Hpx\textsuperscript{-} yaaA cells was similar to that of Hpx\textsuperscript{-} cells in defined medium, approximately 100 \textmu M for both strains at an OD\textsubscript{600} of 0.2 (data not shown).

These results indicate that YaaA suppresses hydroxyl radical formation, and thus the rate of oxidative DNA damage, by diminishing the amount of unincorporated iron inside oxidatively stressed cells.

**YaaA does not repress expression of iron importers.** The factors that control iron import, trafficking, and disposition within the cell are largely mysterious. The best-understood facet of iron regulation is the transcriptional control upon the synthesis of iron importers and of iron-containing enzymes (6, 34). The primary iron importers active in lab cultures of *E. coli* are the ferric enterobactin uptake (Ent) system, the ferric citrate uptake (Fec) system, and the ATP-dependent ferrous import (Feo) system. We observed that Hpx\textsuperscript{-} fec yaaA, Hpx\textsuperscript{-} entA yaaA, and Hpx\textsuperscript{-} feo entA yaaA mutants all exhibited aerobic growth defects compared to their yaaA\textsuperscript{+} counterpart strains (data not shown), which implied that the yaaA phenotype did not depend on a specific iron importer. However, all three systems are controlled at the transcriptional level by the Fur repressor, and it seemed plausible that YaaA might perturb the function of this regulator. More generally, in order to evaluate whether YaaA controls iron levels by controlling transcription of the importer(s), we constructed Δfeo ΔentA derivatives, which rely upon the Fec system as the sole mechanism of iron uptake. Using a fec\textsuperscript{A}-lacZ transcriptional fusion integrated at the lambda attachment site, we found that mutation of the yaaA allele had no impact upon fec\textsuperscript{A} transcription (Fig. 11). Thus, YaaA does not control the expression of iron importers. Other possible points of iron control exist, such as an influence on transporter activity.

**Hpx\textsuperscript{-} mntH yaaA mutants showed a strong growth defect in defined medium.** Our previous data suggested that YaaA helped to control the iron level during peroxide stress; hence, we wanted to check whether YaaA worked with other proteins that also control iron homeostasis during the OxyR response, including the iron uptake regulator (Fur), iron-scavenging protein (Dps), and manganese transporter (MntH). The yaaA deletion exacerbated the growth and viability defects of Hpx\textsuperscript{-} fur and Hpx\textsuperscript{-} dps strains in LB (see Fig. S2 in the supplemental material), but not in defined medium (data not shown). However, Hpx\textsuperscript{-} mntH yaaA mutants showed a strong growth defect in both LB and defined glucose medium (Fig. 12). During H\textsubscript{2}O\textsubscript{2} stress, manganese is imported via MntH to replace the iron cofactor in certain peroxide-sensitive enzymes (2, 45a). In defined medium, Hpx\textsuperscript{-} mntH yaaA cells stopped growing almost immediately after aeration (Fig. 12B), but the number of viable cells remained constant (Fig. 12C). These results suggested that this particular phenotype might be caused by inhibition of metabolism instead of DNA damage. Collectively, these data show that YaaA has functions independent of Fur, Dps, and MntH.

Additional experiments revealed that Hpx\textsuperscript{-} fin and Hpx\textsuperscript{-} hfr mutants grow as well as Hpx\textsuperscript{-} strains, indicating that under our conditions ferritin and bacterioferritin do not play a key role during H\textsubscript{2}O\textsubscript{2} stress and are not involved in the critical function of YaaA (data not shown). Similarly, although FieF is suspected as acting as an iron efflux system (15), an fieF deletion did not diminish the growth of an Hpx\textsuperscript{-} strain, and the further

**FIG. 10. Levels of unincorporated iron are elevated in Hpx\textsuperscript{-} yaaA cells.** The strains were cultured in aerobic LB medium, and intracellular unincorporated iron was determined by whole-cell EPR analysis. Data are reported as the means and standard errors of the means from at least three independent experiments. (A) Cells were harvested at an OD\textsubscript{600} of 0.1, 0.2, and 0.3. (B) Cells were harvested at an OD\textsubscript{600} of 0.2. pWKS30, empty vector; pWK\textsuperscript{yaaA}, plasmid expressing yaaA under control of the lac promoter.

**FIG. 11. fecA\textsuperscript{A}-lacZ induction in Hpx\textsuperscript{-} fec entA strains (yaaA\textsuperscript{+} versus yaaA\textsuperscript{-}).** Cultures were grown in anaerobic LB medium for five generations and either harvested at an OD\textsubscript{600} of 0.2 (-O\textsubscript{2}) or diluted into aerobic LB medium and grown aerobically for five more generations before collection at an OD\textsubscript{600} of 0.2 (+O\textsubscript{2}). The yaaA\textsuperscript{-} strain is Hpx\textsuperscript{-} fec entA fecA\textsuperscript{A}-lacZ (AL273); the yaaA\textsuperscript{+} strain is Hpx\textsuperscript{-} fec entA yaaA fecA\textsuperscript{A}-lacZ (AL293).
addition of a yaaA mutation recapitulated the Hpx− yaaA phenotype (see Fig. S3 in the supplemental material).

Regulation of YaaA. To obtain further insight into the function of YaaA, we measured the expression of the yaaA-lacZ fusion under different conditions (Table 2). Although previous DNA microarray data showed that yaaA was induced 4-fold by treatment with 1 mM H2O2 in an oxyR strain (57), we did not observe this induction in either a β-galactosidase assay or by real-time PCR measurements (data not shown). Our data suggest that OxyR is the sole transcriptional regulator of yaaA in response to oxidative stress.

The expression of yaaA was unaltered (<2-fold induction/repression) under other tested conditions. It did not respond to manganese availability; it was not induced by redox-cycling drugs, which indicates that it is not regulated by SoxR (16, 47); it was not affected by Fur, IscR, Hfq, RpoS, or YaaA itself.

DISCUSSION

In some natural environments bacteria must cope with extended periods of exposure to low levels of H2O2, an experience that was recreated in this study through the use of strains that lack scavenging enzymes. The H2O2 concentration that accumulates in the cytoplasm of aerobic Hpx− mutants, approximately 1 μM, is expected to be reached in the cytoplasm of wild-type cells when they enter environments that contain approximately 5 μM H2O2 (44). The primary effect of these doses upon E. coli is to block growth. The particular damage mechanisms that have been identified thus far all appear to involve Fenton-type reactions between iron and H2O2: the creation of DNA lesions and the inactivation of mononuclear iron enzymes, of iron-sulfur dehydratases, of the Isc Fe/S assembly system, and of the Fur repressor (2, 20, 23, 24, 49). The second-order rate constants for these reactions are high, ca. 103 to 104 M−1 s−1, which means that when H2O2 is present at 1 μM they occur with half-times on a minutes time scale. No other target molecules are known that react with H2O2 so readily, and so it seems fitting that many of the genes controlled by OxyR serve to avoid or to alleviate the damage done by Fenton reactions. YaaA now joins Dps (40, 54) and Fur (49) as a mediator that minimizes the amount of unincorporated iron inside H2O2-stressed cells. Like Dps and Fur, YaaA has the effect of minimizing damage both to DNA and to proteins. Like Dps, but unlike Fur, YaaA seems not to play much of a role during routine growth; it is only during H2O2 stress, when YaaA levels rise, that its influence is apparent. For this reason, yaaA mutants resemble dps mutants in that they do not exhibit unusual sensitivity in experiments that involve a sudden bolus of H2O2; a period of OxyR regulon expression is required for it to become functional. Indeed, mntH and suf mutants only evince a phenotype during protracted, low-grade stress, too (2, 24).

Little is known about the DUF328 family of proteins. They do not have recognizable motifs or absolutely conserved residues, and no crystal structures are available. YaaA homologues are found in only a few eukaryotes, all of which are photosynthetic (four green algae and Ricinus communis). YaaA genes are widely distributed among bacteria, including both anaerobes and aerobes. Their presence in anaerobes does not necessarily indicate that they have a nonoxidative stress role, since even anaerobes are periodically exposed to oxygenated waters and customarily encode antioxidant enzymes, including scavengers of H2O2. A yaaA gene is identifiable in Lactobacillus gasseri, which uses H2O2 as a chemical weapon to inhibit competitors (45b), but we did not find any homologue in Borrelia burgdorferi, a bacterium that seems not to have iron-dependent enzymes or to import iron (42). Thus, the circumstantial data are broadly consistent with a role in iron metabolism and antioxidant activity.

The yaaA phenotype was pronounced in medium that in-

### TABLE 2. Regulation of yaaA

<table>
<thead>
<tr>
<th>Test condition</th>
<th>Mutants (medium used)</th>
<th>Regulator</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O2 stress</td>
<td>Hpx− (LB and defined medium)</td>
<td>OxyR</td>
<td>Yes</td>
</tr>
<tr>
<td>Redox-cycling</td>
<td>WT (LB + 200 μM parquat)</td>
<td>SoxR</td>
<td>No</td>
</tr>
<tr>
<td>Iron rich</td>
<td>fur (LB)</td>
<td>Fur</td>
<td>No</td>
</tr>
<tr>
<td>Iron starved</td>
<td>tonB (defined medium + DFO)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Mn rich</td>
<td>mntR (LB)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Mn starved</td>
<td>mntH (defined medium)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Small RNA</td>
<td>hfg (LB)</td>
<td>Small RNAs</td>
<td>No</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>WT (LB and defined medium)</td>
<td>RpoS</td>
<td>No</td>
</tr>
<tr>
<td>iscR deficient</td>
<td>iscR (LB)</td>
<td>IscR</td>
<td>No</td>
</tr>
<tr>
<td>Autoregulation</td>
<td>Hpx− yaaA (LB)</td>
<td>YaaA</td>
<td>No</td>
</tr>
</tbody>
</table>

* A negative result was defined as a less-than-2-fold change in expression of yaaA.
FIG. 13. Potential mechanisms by which YaaA might diminish levels of unincorporated iron. YaaA might slow down the increase of unincorporated iron by slowing iron import (1) or preventing the leakage of iron from H2O2-damaged enzymes (2), or YaaA might accelerate the decrease of unincorporated iron by sequestering iron (3), speeding iron delivery to target proteins (4), or increasing iron efflux (5).

cluded yeast extract. EPR measurements showed that E. coli generally contains higher levels of unincorporated iron when yeast extract is in the medium. This effect cannot be achieved simply by adding high levels of iron to defined medium, which leads us to suspect that a component of yeast extract makes iron more available for import. Interestingly, yaaA was not as easily induced by H2O2 as was katG. We do not know whether the mechanistic basis of this difference stems from a difference in OxyR affinity, but the end effect resembles the graded responses of members of the SOS regulon, some of which are turned on rapidly and others of which are activated later (41). Thus, catalase synthesis might comprise the first response to slight H2O2 stress, whereas alterations in iron metabolism are engaged only if stress is severe.

So how does YaaA control iron levels? This question is challenging, because the details of iron metabolism remain foggy. In principle, intracellular iron levels might be suppressed in a few ways: by slowing iron import, speeding delivery to iron enzymes, sequestering iron in storage proteins, preventing the leakage of iron from labile enzymes, or pumping excess iron from the cell (Fig. 13). However, YaaA is not an integral membrane protein, meaning that it could at best be an accessory to efflux: it was effective no matter which iron import system was active, it did not seem to affect the damage to Fe/S dehydratases, and it retained a phenotype whether or not cells contained Dps, the primary iron storage system during oxidative stress. Although one can contrive arguments around these facts, they collectively hint that perhaps the remaining option—that YaaA is somehow involved in iron trafficking—is the most likely one. Pull-down experiments did not identify any convincing partner proteins (data not shown). As our understanding of iron metabolism progresses, perhaps a specific hypothesis for the YaaA mechanism will become apparent, as will experimental methods that would test it.

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YaaA AND HYDROGEN PEROXIDE IN E. COLI 2195


