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

Yuanyuan Liu, Sarah C. Bauer, and James A. Imlay*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 2 January 2011/Accepted 26 February 2011

Hydrogen peroxide (H₂O₂) is commonly formed in microbial habitats by either chemical oxidation processes or host defense responses. H₂O₂ can penetrate membranes and damage key intracellular biomolecules, including DNA and iron-dependent enzymes. Bacteria defend themselves against this H₂O₂ 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 H₂O₂ in *Escherichia coli* as part of its OxyR regulon. Here we confirm that *yaaA* is a key element of the stress response to H₂O₂. 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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,

* Corresponding author. Mailing address: Department of Microbiology, University of Illinois at Urbana-Champaign, 601 South Goodwin Ave., Urbana, IL 61801. Phone: (217) 333-5812. Fax: (217) 244-6697. E-mail: jimlay@illinois.edu.

† Supplemental material for this article may be found at <http://jb.asm.org/>.

[∇] Published ahead of print on 4 March 2011.

H₂O₂ is also used as a weapon in a variety of natural antimicrobial systems: plants, amoebae, 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 in mononuclear metal enzymes which otherwise would be damaged by Fenton chemistry (2, 25, 45a).

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 an *Hpx*[−] background. The results indicate that during H₂O₂ stress YaaA prevents oxidative damage to both DNA and proteins by diminishing the amount of unincorporated iron within the cell.

MATERIALS AND METHODS

Reagents. β-Mercaptoethanol, bovine liver catalase, Chelex 100, L-cystine dihydrochloride, deferoxamine mesylate (DFO), diethylenetriaminepentaacetic acid (DTPA), ferric chloride, type II horseradish peroxidase, 30% hydrogen peroxide, o-nitrophenyl-β-D-galactopyranoside (ONPG), thiamine, and trimethoprim (TMP) were purchased from Sigma. Potassium cyanide and α-(4-pyridyl-1-oxide)-N-tert-butyl nitron (4-POBN) were from Aldrich. Hanks balanced salt solution without CaCl₂, MgCl₂, MgSO₄, and phenol red (HBSS) was obtained from Gibco. Hy-Case Amino and 2,4-dinitrophenylhydrazine (DNPH) were from Fluka, and Amplex Red was purchased from Molecular Probes. Standard reagents for RNA work were obtained from Ambion. iQ SYBR Green supermix was from Bio-Rad, and real-time PCR plates were purchased from Eppendorf.

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.

Plasmid construction. The *yaaA* coding sequence was amplified by PCR from *E. coli* MG1655 genomic DNA using the forward primer 5'-TTTCAGAATTC TAAATTCCTGCAAGGACTG-3' and the reverse primer 5'-GATCCTCTA GACAACTTAACGCTGCTCGTA-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.

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 autoclaving 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.

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.

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).

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 catalase to stop oxidative stress, and washed with minimal glucose/amino acids medium at room temperature three times. The cells were placed 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. *thyA* 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 colonies was tested and confirmed to be Thy[−] by examining their growth on minimal glucose/amino acids plates with and without thymidine supplementation.

H₂O₂ 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.

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,

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Source or reference
<i>E. coli</i> strains		
AA30	As LC106 plus Δ mntH2::cat	2
AL145	As LC106 plus Δ (lacZ1::cat)1 attλ::[pSJ501::yaaA'-lacZ ⁺]-cat	This study
AL154	As AL157 plus Δ (ahpC-ahpF') kan::'ahpF	This study
AL157	As MG1655 plus Δ (lacZ1::cat)1 attλ::[pSJ501::yaaA'-lacZ ⁺]-cat	This study
AL194	As LC106 plus Δ (yaaA1::cat)1 fur::kan zbf-507::Tn10	This study
AL221	As LC106 plus Δ (yaaA1::cat)1 Δ mntH2::cat	This study
AL223	As MG1655 plus recA56 srl300::Tn10 Δ (yaaA1::cat)1	This study
AL245	As MG1655 plus Δ (yaaA1::cat)1	This study
AL273	As LC106 plus Δ (feoABC::cat)1 Δ entA1::cat Δ (lacZ1::cat)1 attλ::[pSJ501::fecA'-lacZ ⁺]-cat	This study
AL285	As LC106 plus Δ (yaaA1::cat)1 Δ dps::cat	This study
AL293	As AL273 plus Δ (yaaA1::cat)1	This study
AL344	As AL145 plus Δ oxyR::spec	This study
AL435	As LC106 plus Δ fieF1::cat	This study
AL437	As LC106 plus Δ (yaaA1::cat)1 Δ fieF1::cat	This study
BW25113	lacI ^r rrmB _{T14} lacZ _{WJ16} hsdR514 araBAD _{AH33} rhaBAD _{LD78}	10
LC106	As MG1655 plus Δ (ahpC-ahpF') kan::'ahpF Δ (katG17::Tn10)1 Δ (katE12::Tn10)1	45
LEM17	As MG1655 plus recA56 srl300::Tn10	Laboratory stock
MG1655	Wild type	<i>E. coli</i> Genetic Stock Center
SB53	As LC106 plus Δ (yaaA1::cat)1	This study
SP66	As LC106 plus mhpC281::Tn10 lacY1 dps::cat	40
Plasmids		
pAH125	Plasmid for lacZ transcriptional fusion construction	18
pCP20	Temperature-sensitive Flp expression plasmid	9
pKD3	FRT-flanked cat	10
pKD46	λ-Red recombinase expression plasmid	10
pACYC184	Cm ^r Tet ^r	8
pGSO58	oxyR A233V in pACYC184	29
pSJ501	pAH125 derivative with FRT-flanked cat	24
pWKS30	P _{lac} polylinker Amp ^r	50
pWkyaaA	pWKS30 containing yaaA insert	This study

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 (OD₆₀₀ 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. Cells were centrifuged 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 iron 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 (pH 7.4) and finally resuspended in 200 μl of ice-cold 20 mM Tris-HCl-30% glycerol (pH 7.4). The EPR analysis was performed as described previously (33). The measured EPR signals were normalized by cell density and converted to intracellular concentrations by using the following conversion: 1 ml of *E. coli* culture at an OD₆₀₀ of 1 comprises 0.52 μl of intracellular volume (21).

β-Galactosidase activity. Cell extracts were prepared in ice-cold 50 mM Tris-HCl (pH 8.0) by passage through a French pressure cell (SIM Aminco FA-003). Cell debris was removed by centrifugation, and β-galactosidase activity in the supernatant was measured by standard procedures (37).

Protein concentrations were determined by the Bradford assay (Coomassie protein assay reagent; Thermo Scientific) using bovine serum albumin (albumin standard; Thermo Scientific) as the standard.

H₂O₂ measurement. Cells were cultured in defined glucose/amino acids medium under anaerobic conditions to log phase, and then they were diluted into aerobic medium to an OD₆₀₀ of 0.005 and grown to an OD₆₀₀ of ~0.15. These cells were centrifuged, suspended in fresh defined medium at an OD₆₀₀ of 0.020, and incubated aerobically at 37°C with vigorous aeration. At time points cells were removed by centrifugation, and H₂O₂ concentrations were measured in the supernatants by using the Amplex Red/horseradish peroxidase method (43) in a Shimadzu RF Mini-150 fluorometer.

Visualization of protein carbonylation. Cells were cultured in aerobic LB as described above. Hpx⁻ yaaA cells were harvested by centrifugation at the beginning (OD₆₀₀, 0.2) and in the middle of the growth lag (OD₆₀₀, ~0.25). Other strains were collected at an OD₆₀₀ 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 DNPH, and the adducts were visualized by Western blotting (2).

Rapid amplification of cDNA ends (5'-RACE). MG1655 cells and Hpx⁻ cells were cultured in LB as described above and harvested at an OD₆₀₀ 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 outer 5'-race, 5'-CTCGTTTCAGCTTGTTG GTGAT-3', and yaaA inner 5'-race, 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 OD₆₀₀ of ~0.2, they were treated with H₂O₂ (0, 0.25, 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'-GCTGTTAGACAAT TCCCAGCAG-3'; yaaA reverse, 5'-AAGCCGGTGTAGACATCACCTT-3'; gapA forward, 5'-CGTTCTGGGCTACCCGAAGATGACG-3'; gapA reverse, 5'-AAGCCGGTTTCGTTGTCGTACCAGGA-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

FIG. 1. Genomic context of *yaaA* in *E. coli*. In *E. coli*, *yaaA* is located at 0.12 min and is flanked by two genes with undefined functions, *yaaJ* and *yaaX*. The genomic context for *yaaA* is not conserved among bacteria that possess this gene, and no obvious gene partners were found by computational analyses. A computational search using an OxyR consensus sequence (58) identified one potential OxyR binding site (bp -74 to -38), and 5'-RACE analysis mapped the 5' end of *yaaA* transcripts to 22 bp upstream of the *yaaA* start codon. TSS, transcriptional start site.

apparatus (Eppendorf) for 40 cycles in three steps: 95°C for 20 s, 55°C for 20 s, and 68°C for 20 s.

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 (Hpx⁻), which accumulates up to ~1 μM endogenous H₂O₂ and in which the OxyR response is fully activated (43) (Fig. 2A). Induction did not occur if an *oxyR* deletion was present (Fig. 2B). 5'-RACE revealed that the 5' end of *yaaA* transcripts were identical for both wild-type cells and Hpx⁻ cells, mapping to 22 bp upstream of the *yaaA* start codon (Fig. 1). When wild-type cells were transformed with the plasmid pGSO58, which contains a mutated *oxyR* allele (A233V) that constitutively expresses 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 *ahp*-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

FIG. 2. A transcriptional *yaaA'-lacZ* fusion confirms that *yaaA* is part of the OxyR regulon. (A) Cultures were grown in anaerobic or aerobic LB medium for five generations before collection and β-galactosidase measurements (OD₆₀₀, ~0.2). WT, MG1655; vector, empty vector control (pACYC184); pGSO58, plasmid-borne mutant OxyR A233V that constitutively overexpresses OxyR-regulated genes; *ahp*⁻ (AL154), 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 OD₆₀₀s 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.

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.

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; Δ *yaaA* (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).

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*⁻ strains can gradually degrade H₂O₂ that is found in LB medium, because the H₂O₂ is a weak substrate for the cytochrome oxidases of the respiratory chain (A. L. Woodmansee and J. A. Imlay, unpublished

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).

data); a similar recovery has been observed in *Hpx*⁻ strains that lack *fur* or *dps*, two other genes that are critical during H₂O₂ 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 H₂O₂ stress.** Previous work has shown that H₂O₂ directly inactivates enzymes that employ solvent-exposed iron atoms either in iron-sulfur clusters or as mononuclear cofactors (23, 45a). Further, H₂O₂ 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. 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. 6. Aerobic mutation frequencies are elevated in the *Hpx*⁻ *yaaA* mutant compared to its *Hpx*⁻ parent strain. Exponentially growing cells in anaerobic LB medium were diluted into aerobic LB medium, and the mutation frequencies were measured at subsequent time points as described in Materials and Methods. The zero time point data were obtained immediately prior to aeration. The mutation frequencies in *Hpx*⁺ strains (barely visible above) were about 0.5 to 1 mutants/10⁶ cells.

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 H₂O₂ 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 H₂O₂ 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

FIG. 7. *Hpx*⁺ *recA yaaA* mutants exhibited growth and viability defects. Anaerobic exponential-phase cultures were diluted into aerobic LB medium at time zero. (A, C, and D) Growth rates, based on the optical density. The *recA yaaA* (AL223) mutants exhibited a growth (A) and viability (B) defect that was suppressed by ~50 U/ml catalase (C) or 2 mM DFO (D).

FIG. 8. Protein carbonylation in *Hpx*⁻ *yaaA* cells. Cells were cultured in aerobic LB medium as described in Materials and Methods. The *Hpx*⁻ *yaaA* cells were harvested at the beginning (OD₆₀₀, 0.2; 3 h) and in the middle of the growth lag (6 h). Other strains were collected at an OD₆₀₀ of ~0.2. Strains used were as follows: wild type (WT; MG1655); *Hpx*⁻ *dps* (SP66), a positive control for carbonylation; *Hpx*⁻ (LC106); *Hpx*⁻ *yaaA* (SB53).

levels of H₂O₂ 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 H₂O₂ 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*⁻ *yaaA* and *Hpx*⁻ *yaaA*⁺ mutants when they were grown in defined glucose medium (data not shown). However, when they were cultured in aerobic LB medium, the *Hpx*⁻ *yaaA* 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 H₂O₂ stress. The activities of ribulose-5-phosphate epimerase and isopropylmalate isomerase, respectively, were compared in *Hpx*⁻ and *Hpx*⁻ *yaaA* 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 H₂O₂ 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

FIG. 9. The $\text{Hpx}^- \text{yaaA}$ mutant exhibited higher levels of hydroxyl radicals. Cells were cultured in aerobic LB as described earlier in this report, and they were harvested at an OD_{600} of 0.2, which corresponded to the beginning of the growth lag of $\text{Hpx}^- \text{yaaA}$ cells. The final cell densities were equivalent in all samples. The six peaks represented the $\text{HO}^\bullet$ signal of Hpx^- cells and $\text{Hpx}^- \text{yaaA}$ cells.

siderophore and ferric-citrate systems (51) (Fig. 3B). Furthermore, DFO also eliminated the growth defect of *recA yaaA* mutants (Fig. 7D). These results confirm that the key injury in the *yaaA* mutant during H_2O_2 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 $\text{HO}^\bullet$ 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 $\text{HO}^\bullet$ formation.

Intracellular $\text{HO}^\bullet$ 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^- mutants, as reported previously (40). In the early period after aeration, the $\text{Hpx}^- \text{yaaA}$ cells exhibited a signal similar to that of Hpx^- cells (data not shown). However, the signal continually increased in the $\text{Hpx}^- \text{yaaA}$ cells, ultimately becoming about twice that of the Hpx^- strain (Fig. 9). Pretreatment with 2 mM dipyridyl (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 H_2O_2 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; $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{HO}^\bullet$) followed by the regeneration of ferrous iron mediated by cellular reductants (reaction 2; $\text{Fe}^{3+} + \text{red} \rightarrow \text{Fe}^{2+} + \text{ox}$).

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 H_2O_2 , unincorporated iron, and cysteine/flavin. The spin-trapping data

suggested that YaaA suppresses the level of one of these reactants.

The rate of intracellular H_2O_2 formation depends upon the titers of autoxidizable enzymes in the cell, and in Hpx^- strains the steady-state level of H_2O_2 also depends upon minor scavenging mechanisms. We sought to test whether YaaA might affect either of these processes. In Hpx^- strains the H_2O_2 concentration rapidly equilibrates between the cytoplasm and the external medium; therefore, cocultured Hpx^- strains experience equivalent intracellular concentrations of H_2O_2 , and a strain that efficiently scavenges H_2O_2 can suppress the growth defects that would otherwise arise in a cocultured strain that cannot (44). To this end, we cocultured Hpx^- cells and $\text{Hpx}^- \text{yaaA}$ 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 $\text{Hpx}^- \text{yaaA}$ strain continued to exhibit its full aerobic viability defect, while the Hpx^- strain did not. The result indicates that YaaA does not alter the intracellular levels of H_2O_2 (data not shown).

The rate of cellular H_2O_2 formation was also directly measured as described in Materials and Methods. These experiments were performed in defined media, as components in LB interfere with H_2O_2 measurements. Under these conditions, the rate of H_2O_2 accumulation in Hpx^- cells was 11 ± 1 nM/min (mean $\pm$ standard error of the mean), versus 11 ± 0.3 nM/min in $\text{Hpx}^- \text{yaaA}$ cells, which further indicates that YaaA does not affect the rate of H_2O_2 accumulation.

YaaA does not inhibit ferric iron reduction. FADH_2 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 H_2O_2 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/ H_2O_2 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 H_2O_2 sensitivity (39). These results suggest that YaaA is unlikely to attenuate the Fenton reaction by inhibiting the rate of ferric iron reduction (reaction 2).

$\text{Hpx}^- \text{yaaA}$ mutants have higher unincorporated iron levels than the Hpx^- 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_{600} of 0.1, 0.2, and 0.3; these time points represented $\text{Hpx}^- \text{yaaA}$ cells prior to the growth lag, at its onset, and at its midpoint. The data showed that deletion of *yaaA* in unstressed Hpx^+ cells had no effect on the iron levels of approximately 65 μM (Fig. 10A). The level of unincorporated iron in H_2O_2 -stressed Hpx^- 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 $\text{Hpx}^- \text{yaaA}$ cells consistently accumulated 2-fold-higher levels of unincorporated iron (300 μM) by the begin-

FIG. 10. Levels of unincorporated iron are elevated in Hpx⁻ *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 OD₆₀₀ of 0.1, 0.2, and 0.3. (B) Cells were harvested at an OD₆₀₀ of 0.2. pWKS30, empty vector; pWkyaaA, plasmid expressing *yaaA* under control of the *lac* promoter.

ning (OD₆₀₀, 0.2) of the growth lag (Fig. 10A). This effect matches the 2-fold effect of *yaaA* upon hydroxyl radical formation. The iron returned to Hpx⁻ *yaaA*⁺ levels when *yaaA* was expressed in *trans* on a plasmid (Fig. 10B).

Hpx⁻ *yaaA* cells do not show a growth defect compared to Hpx⁻ 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⁻ *yaaA* cells was similar to that of Hpx⁻ cells in defined medium, approximately 100 μM for both strains at an OD₆₀₀ 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⁻ *fec yaaA*, Hpx⁻ *entA yaaA*, and Hpx⁻ *feo entA yaaA* mutants all exhibited

FIG. 11. *fecA'*-*lacZ* induction in Hpx⁻ *feo entA* strains (*yaaA*⁺ versus *yaaA*⁻). Cultures were grown in anaerobic LB medium for five generations and either harvested at an OD₆₀₀ of ~0.2 (-O₂) or diluted into aerobic LB medium and grown aerobically for five more generations before collection at an OD₆₀₀ of ~0.2 (+O₂). The *yaaA*⁺ strain is Hpx⁻ *feo entA fecA'*-*lacZ* (AL273); the *yaaA*⁻ strain is Hpx⁻ *feo entA yaaA fecA'*-*lacZ* (AL293).

aerobic growth defects compared to their *yaaA*⁺ 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 Δ *feo* Δ *entA* derivatives, which rely upon the Fec system as the sole mechanism of iron uptake. Using a *fecA'*-*lacZ* transcriptional fusion integrated at the lambda attachment site, we found that mutation of the *yaaA* allele had no impact upon *fecA* 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⁻ *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⁻ *fur* and Hpx⁻ *dps* strains in LB (see Fig. S2 in the supplemental material), but not in defined medium (data not shown). However, Hpx⁻ *mntH yaaA* mutants showed a strong growth defect in both LB and defined glucose medium (Fig. 12). During H₂O₂ stress, manganese is imported via MntH to replace the iron cofactor in certain peroxide-sensitive enzymes (2, 45a). In defined medium, Hpx⁻ *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⁻ *fm* and Hpx⁻ *bfr* mutants grow as well as Hpx⁻ strains, indicating that under our conditions ferritin and bacterioferritin do not play a key role during H₂O₂ 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⁻ strain, and the further

FIG. 12. $Hpx^- mntH yaaA$ cells exhibited growth defects in both LB and defined glucose medium. Anaerobic exponential-phase cultures were diluted into the same aerobic medium at time zero. Strains used included the following: Hpx^- (LC106), $Hpx^- mntH$ (AA30), $Hpx^- yaaA$ (SB53), and $Hpx^- mntH yaaA$ (AL221). (A) Optical density in LB. (B and C) Optical density (B) and cell viability (C) in defined glucose medium.

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 H_2O_2 in an $\Delta oxyR$ 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 H_2O_2 , an experience that was recreated in this study through the use of strains that lack scavenging enzymes. The H_2O_2 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 H_2O_2 (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 H_2O_2 : 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. 10^3 to 10^4 $M^{-1} s^{-1}$, which means that when H_2O_2 is present at 1 μM they occur with half-times on a minutes time scale. No other target molecules are known that react with H_2O_2 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 H_2O_2 -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 H_2O_2 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 H_2O_2 ; 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 H_2O_2 . A *yaaA* gene is identifiable in *Lactobacillus gasseri*, which uses H_2O_2 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*

Test condition	Mutants (medium used)	Regulator	Result ^a
H_2O_2 stress	Hpx^- (LB and defined medium)	OxyR	Yes
Redox-cycling drug	WT (LB + 200 μM paraquat)	SoxR	No
Iron rich	<i>fur</i> (LB)	Fur	No
Iron starved	<i>tonB feo</i> (defined medium + DFO)		No
Mn rich	<i>mntR</i> (LB)		No
Mn starved	<i>mntH</i> (defined medium)		No
Small RNA	<i>hfq</i> (LB)	Small RNAs	No
Stationary phase	WT (LB and defined medium)	RpoS	No
<i>iscR</i> deficient	<i>iscR</i> (LB)	IscR	No
Autoregulation	$Hpx^- yaaA$ (LB)	YaaA	No

^a 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.

ACKNOWLEDGMENTS

We thank Ian Gut (University of Illinois) for assistance with DIC microscopy, Jason Sobota (University of Illinois) for assistance with

the ribulose-5-phosphate epimerase assay, and Mark Nilges (Illinois EPR Research Center) for assistance with EPR experiments.

This work was supported by GM049640 from the National Institutes of Health.

REFERENCES

- Ananthaswamy, H. N., and A. Eisenstark. 1977. Repair of hydrogen peroxide-induced single-strand breaks in *Escherichia coli* deoxyribonucleic acid. *J. Bacteriol.* **130**:187–191.
- Anjem, A., S. Varghese, and J. A. Imlay. 2009. Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*. *Mol. Microbiol.* **72**:844–858.
- Aslund, F., M. Zheng, J. Beckwith, and G. Storz. 1999. Regulation of the OxyR transcriptional factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc. Natl. Acad. Sci. U. S. A.* **96**:6161–6165.
- Bedard, K., B. Lardy, and K.-H. Krause. 2007. NOX family NADPH oxidases: not just in mammals. *Biochimie* **89**:1107–1112.
- Bjerrum, C. J., and D. E. Canfield. 2002. Ocean productivity before about 1.9 Gyr ago limited by phosphorus adsorption onto iron oxides. *Nature* **417**:159–162.
- Braun, V. 2003. Iron uptake by *Escherichia coli*. *Front. Biosci.* **1**:s1409–s1421.
- Carliz, A., and D. Touati. 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? *EMBO J.* **5**:623–630.
- Chang, A. C., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**:1141–1156.
- Cherepanov, P. P., and W. Wackernagel. 1995. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**:9–14.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**:6640–6645.
- Finn, R. D., et al. 2010. The Pfam protein families database. *Nucleic Acids Res.* **38**:D211–D222.
- Flint, D. H., J. F. Tuminello, and M. H. Emptage. 1993. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J. Biol. Chem.* **268**:22369–22376.
- Glass, G. A., et al. 1986. The respiratory burst oxidase of human neutrophils. Further studies of the purified enzyme. *J. Biol. Chem.* **261**:13247–13251.
- Grant, R. A., D. J. Filman, S. E. Finkel, R. Kolter, and J. M. Hogle. 1998. The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nat. Struct. Biol.* **5**:294–303.
- Grass, G., et al. 2005. FieF (YiiP) from *Escherichia coli* mediates decreased cellular accumulation of iron and relieves iron stress. *Arch. Microbiol.* **183**:9–18.
- Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Dimple. 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **87**:6181–6185.
- Gut, I. M., A. M. Prouty, J. D. Ballard, W. A. van der Donk, and S. R. Blanke. 2008. Inhibition of *Bacillus anthracis* spore outgrowth by nisin. *Antimicrob. Agents Chemother.* **52**:4281–4288.
- Haldimann, A., and B. L. Wanner. 2001. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. *J. Bacteriol.* **183**:6384–6393.
- Ilari, A., P. Ceci, D. Ferrari, G. Rossi, and E. Chiancone. 2002. Iron incorporation into *Escherichia coli* Dps gives rise to a ferritin-like microcrystalline core. *J. Biol. Chem.* **277**:37619–37623.
- Imlay, J. A., S. M. Chin, and S. Linn. 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* **240**:640–642.
- Imlay, J. A., and I. Fridovich. 1991. Assay of metabolic superoxide production in *Escherichia coli*. *J. Biol. Chem.* **266**:6957–6965.
- Inbaraj, J. J., and C. F. Chignell. 2004. Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes. *Chem. Res. Toxicol.* **17**:55–62.
- Jang, S., and J. A. Imlay. 2007. Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. *J. Biol. Chem.* **282**:929–937.
- Jang, S., and J. A. Imlay. 2010. Hydrogen peroxide inactivates the *Escherichia coli* Isc iron-sulfur assembly system, and OxyR induces the Suf system to compensate. *Mol. Microbiol.* **78**:1448–1467.
- Kehres, D. G., A. Janakiraman, J. M. Schlauch, and M. E. Maguire. 2002. Regulation of *Salmonella enterica* serovar Typhimurium *mntH* transcription by H₂O₂, Fe²⁺, and Mn²⁺. *J. Bacteriol.* **184**:3151–3158.
- Keyer, K., and J. A. Imlay. 1996. Superoxide accelerates DNA damage by elevating free-iron levels. *Proc. Natl. Acad. Sci. U. S. A.* **93**:13635–13640.
- Korshunov, S., and J. A. Imlay. 2006. Detection and quantification of superoxide formed within the periplasm of *Escherichia coli*. *J. Bacteriol.* **188**:6326–6334.

28. Korshunov, S., and J. A. Imlay. 2010. Two sources of endogenous hydrogen peroxide in *Escherichia coli*. *Mol. Microbiol.* **75**:1389–1401.
29. Kullik, I., M. B. Toledano, L. A. Tartaglia, and G. Storz. 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. *J. Bacteriol.* **177**:1275–1284.
30. Kuo, C. F., T. Mashino, and I. Fridovich. 1987. α,β -Dihydroxyisovalerate dehydratase: a superoxide-sensitive enzyme. *J. Biol. Chem.* **262**:4724–4727.
31. Lee, C., et al. 2004. Redox regulation of OxyR requires specific disulfide bond formation involving a rapid kinetic reaction path. *Nat. Struct. Mol. Biol.* **11**:1179–1185.
32. Lee, J. H., W. S. Yeo, and J. H. Roe. 2004. Induction of the *sufA* operon encoding Fe-S assembly proteins by superoxide generators and hydrogen peroxide: involvement of OxyR, IHF and an unidentified oxidant-responsive factor. *Mol. Microbiol.* **51**:1745–1755.
33. Macomber, L., C. Rensing, and J. A. Imlay. 2007. Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli*. *J. Bacteriol.* **189**:1616–1626.
34. Masse, E., H. Salvail, G. Desnoyers, and M. Arguin. 2007. Small RNAs controlling iron metabolism. *Curr. Opin. Microbiol.* **10**:140–145.
35. Mehdy, M. C. 1994. Active oxygen species in plant defense against pathogens. *Plant Physiol.* **105**:467–472.
36. Messner, K. R., and J. A. Imlay. 1999. The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. *J. Biol. Chem.* **274**:10119–10128.
37. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
38. Outten, F. W., O. Djaman, and G. Storz. 2004. A *suf* operon requirement for Fe-S cluster assembly during iron starvation in *Escherichia coli*. *Mol. Microbiol.* **52**:861–872.
39. Park, S., and J. A. Imlay. 2003. High levels of intracellular cysteine promote oxidative DNA damage by driving the Fenton reaction. *J. Bacteriol.* **185**:1942–1950.
40. Park, S., X. You, and J. A. Imlay. 2005. Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx- mutants of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **102**:9317–9322.
41. Patel, M., Q. Jiang, R. Woodgate, M. M. Cox, and M. F. Goodman. 2010. A new model for SOS-induced mutagenesis: how RecA protein activates DNA polymerase V. *Crit. Rev. Biochem. Mol. Biol.* **45**:171–184.
42. Posey, J. E., and F. C. Gherardini. 2000. Lack of a role for iron in the Lyme disease pathogen. *Science* **288**:1651–1653.
43. Seaver, L. C., and J. A. Imlay. 2001. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J. Bacteriol.* **183**:7173–7181.
44. Seaver, L. C., and J. A. Imlay. 2001. Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*. *J. Bacteriol.* **183**:7182–7189.
45. Seaver, L. C., and J. A. Imlay. 2004. Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? *J. Biol. Chem.* **279**:48742–48750.
- 45a. Sobota, J. M., and J. A. Imlay. 14 March 2011, posting date. Iron enzyme ribulose-5-phosphate 3-epimerase in *Escherichia coli* is rapidly damaged by hydrogen peroxide but can be protected by manganese. *Proc. Natl. Acad. Sci. U. S. A.* doi:10.1073/pnas.1100410108.
- 45b. Song, Y.-L., et al. 1999. Identification of and hydrogen peroxide production by fecal and vaginal lactobacilli isolated from Japanese women and newborn infants. *J. Clin. Microbiol.* **37**:3062–3064.
46. Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Despiéd. 1995. Lethal oxidative damage and mutagenesis are generated by iron in delta *fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. *J. Bacteriol.* **177**:2305–2314.
47. Tsaneva, I. R., and B. Weiss. 1990. *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. *J. Bacteriol.* **172**:4197–4205.
48. Turner, J. M., and A. J. Messinger. 1986. Occurrence, biochemistry, and physiology of phenazine pigment production. *Adv. Microb. Physiol.* **27**:211–275.
49. Varghese, S., A. Wu, S. Park, K. R. C. Imlay, and J. A. Imlay. 2007. Submicromolar hydrogen peroxide disrupts the ability of Fur protein to control free-iron levels in *Escherichia coli*. *Mol. Microbiol.* **64**:822–830.
50. Wang, R. F., and S. R. Kushner. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**:195–199.
51. Wiener, M. C. 2005. TonB-dependent outer membrane transport: going for baroque? *Curr. Opin. Struct. Biol.* **15**:294–400.
52. Woodmansee, A. L., and J. A. Imlay. 2002. Quantitation of intracellular free iron by electron paramagnetic resonance spectroscopy. *Methods Enzymol.* **349**:3–9.
53. Woodmansee, A. N., and J. A. Imlay. 2002. Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. *J. Biol. Chem.* **277**:34055–34066.
54. Zhao, G., et al. 2002. Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. *J. Biol. Chem.* **277**:27689–27696.
55. Zheng, M., F. Aslund, and G. Storz. 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279**:1718–1721.
56. Zheng, M., B. Doan, T. D. Schneider, and G. Storz. 1999. OxyR and SoxRS regulation of *fur*. *J. Bacteriol.* **181**:4639–4643.
57. Zheng, M., et al. 2001. DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J. Bacteriol.* **183**:4562–4570.
58. Zheng, M., et al. 2001. Computation-directed identification of OxyR DNA binding sites in *Escherichia coli*. *J. Bacteriol.* **183**:4571–4579.