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Alkyl Hydroperoxide Reductase Is the Primary Scavenger of Endogenous Hydrogen Peroxide in *Escherichia coli*

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Hydrogen peroxide is generated during aerobic metabolism and is capable of damaging critical biomolecules. However, mutants of *Escherichia coli* that are devoid of catalase typically exhibit no adverse phenotypes during growth in aerobic media. We discovered that catalase mutants retain the ability to rapidly scavenge H_2O_2 whether it is formed internally or provided exogenously. Analysis of candidate genes revealed that the residual activity is due to alkyl hydroperoxide reductase (Ahp). Mutants that lack both Ahp and catalase could not scavenge H_2O_2. These mutants excreted substantial amounts of H_2O_2, and they grew poorly in air. Ahp is kinetically a more efficient scavenger of trace H_2O_2 than is catalase and therefore is likely to be the primary scavenger of endogenous H_2O_2. Accordingly, mutants that lack Ahp accumulated sufficient hydrogen peroxide to induce the OxyR regulon, whereas the OxyR regulon remained off in catalase mutants. Catalase still has an important role in wild-type cells, because the activity of Ahp is saturated at a low (10^{-5} M) concentration of H_2O_2. In contrast, catalase has a high K_m, and it therefore becomes the predominant scavenger when H_2O_2 concentrations are high. This arrangement is reasonable because the cell cannot provide enough NADH for Ahp to rapidly degrade large amounts of H_2O_2. In sum, *E. coli* does indeed generate substantial H_2O_2, but damage is averted by the scavenging activity of Ahp.

Virtually all aerobic organisms contain enzymes that convert superoxide and hydrogen peroxide to innocuous products. The ubiquity of these scavenging enzymes suggests that exposure to O_2^- and H_2O_2 is an inevitable part of the aerobic lifestyle and that these species can damage cells. It follows that organisms that cannot scavenge them will fare poorly in an aerobic habitat, and in 1971 McCord et al. proposed that some obligate anaerobes may be unable to grow in air at least in part because they lack sufficient levels of scavenging enzymes (17). This reasoning also suggested that aerobes would be much less oxygen tolerant if they lacked superoxide dismutase (SOD) and catalase. In 1985 this prediction was partly affirmed by studies of *sodA sodB* mutants of *Escherichia coli* (3). These strains suffered elevated rates of DNA damage, could not catabolize nonfermentable carbon sources, and did not grow at all without extensive amino acid supplements. However, contrary to expectation, mutants that lacked catalase grew as well as their wild-type parents and exhibited no increase in mutation rate (14, 29).

The fitness of the catalase mutants did not reflect invulnerability to H_2O_2. Low concentrations of exogenous H_2O_2 (ca. 30 μM) are sufficient to inhibit the growth of *E. coli*. Although the growth-blocking cell lesions have not yet been identified, H_2O_2 can oxidize thiols, which may inactivate enzymes that have active-site sulfhydryl residues. Methionine sulfoxide adducts (21) and protein carbonyls (5) may also be products of enzyme oxidation. In addition, H_2O_2 can inactivate the exposed [4Fe-4S] clusters of aconitase B and fumarase B (S. M. Varghese, S. Korshunov, and J. A. Imlay, unpublished data). Finally, reactions between H_2O_2 and intracellular iron generate hydroxyl radicals, which in turn attack DNA. Micromolar concentrations of H_2O_2 are therefore mutagenic.

Thus, the robust performance of catalase mutants seemed to imply that these amounts of H_2O_2 are not normally generated during aerobic growth. There are presently no data that firmly establish the amount of H_2O_2 that is formed as a by-product of metabolism. No enzymes in *E. coli*, other than SOD, generate H_2O_2 as a deliberate, stoichiometric product. However, in vitro studies have shown that H_2O_2 can be formed by the adventitious oxidation of redox enzymes by molecular oxygen.

The main sources of H_2O_2 inside the cell are probably flavoenzymes, both because they are abundant and because flavins are amenable to the univalent electron transfer reaction that initiates the production of superoxide and H_2O_2 (6, 18). The rates at which some flavoenzymes generate these species have been measured in vitro, and extrapolations suggest that they may form about 10 μM H_2O_2 s^{-1} in vivo (18). Gonzalez-Flecha and Demple reported evidence of endogenous H_2O_2 production by *E. coli* (7). If these rates are correct, then the lack of phenotype of catalase mutants seems difficult to explain.

In this study, we discovered that mutants which are devoid of catalase still scavenge physiological concentrations of H_2O_2 as rapidly as do wild-type cells. By identifying and eliminating the remaining scavenging enzyme, alkyl hydroperoxide reductase, we were able to directly quantify endogenous H_2O_2 production and demonstrate that it is enough to poison scavengerless cells.

MATERIALS AND METHODS

Chemicals, enzymes, and media. Cumene hydroperoxide, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), horseradish peroxidase (type II), hydrogen peroxide, *o*-dianisidine, *o*-nitrophenyl-β-D-galactopyranoside, plumbagin, and potassium cyanide were purchased from Sigma. Coomassie protein reagent was obtained from Pierce. Bovine liver catalase (20 mg/ml) was from Boehringer Mannheim, and Amplex red (AR) was from Molecular Probes. Dimethyl sul-

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TABLE 1. *E. coli* strains

Strain	Genotype	Source or reference
UM1	*katE katG14 lacY rspL thi-1*	14
UM120	*katE12*::Tn*10 hfrH thi-1*	Peter Loewen
UM202	*katG17*::Tn*10 hfrH thi-1*	Peter Loewen
GK100	Δ*cydAB*::*cam* Δ(*cyoABCDE*)*456*::*kan*	12
KM38	As UM1 plus Δ*cydAB*::*cam*	P1(GK100) × UM1
KM39	As UMI plus Δ(*cyoABCDE*)*456*::*kan*	P1(GK100) × UM1
SK2255	*zbe-279*::Tn*10 thyA6 rps120 decC1*	*E. coli* Genetic Stock Center
N9716	As GC4468 plus Δ*oxyR*::*spec*	Gisela Storz
AS430	Δ*oxyR*::*spec* Δ*lacU169 rpsL*	P1(N9716) × GC4468
AB1157	F^- *thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rspL supE44 ara-14 xyl-15 mtl-1 tsx-33*	10
MG1655	F^- wild-type	*E. coli* Genetic Stock Center
JI360	As MG1655 plus *katE12*::Tn*10*	P1(UM120) × MG1655
JI361	As MG1655 plus *katG17*::Tn*10*	P1(UM202) × MG1655
JI362	As JI360 plus Δ(*katE12*::Tn*10*)*1* (Tet^s)	Tet^s derivative of JI360
JI364	As JI361 plus Δ(*katG17*::Tn*10*)*1* (Tet^s)	Tet^s derivative of JI361
JI367	As JI364 plus *katE12*::Tn*10*	P1(UM120) × JI364
JI370	As MG1655 plus Δ*ahpCF′ kan*::*′ahpF*	P1(MC4100ΔahpCF) × MG1655
JI372	As JI362 plus Δ*ahpCF′ kan*::*′ahpF*	P1(MC4100ΔahpCF) × JI362
JI374	As JI364 plus Δ*ahpCF′ kan*::*′ahpF*	P1(MC4100ΔahpCF) × JI364
JI377	As JI367 plus Δ*ahpCF′ kan*::*′ahpF*	P1(MC4100ΔahpCF) × JI367
MC4100	*araD139* Δ(*argF-lac*)*169* λ^- *flhD5301 fruA25 relA1 rpsL150 rbsR22 deoC1*	*E. coli* Genetic Stock Center
MC4100ΔahpCF	As MC4100 plus Δ*ahpCF′ kan*::*′ahpF*	Gisela Storz
GS022	As MC4100 plus λRS45 Φ(*katG*::*lacZ*)	Gisela Storz
LC70	As GS022 plus Δ*ahpCF′ kan*::*′ahpF*	P1(MC4100ΔahpCF) × GS022
LC74	As LC70 plus Δ*oxyR*::*spec*	P1(AS430) × LC70
LC80	As GS022 plus *katG17*::Tn*10*	P1(UM202) × GS022
HDO3	As GS022 plus *katE12*::Tn*10*	P1(UM120) × GS022
LC82	As LC80 plus Δ(*katG17*::Tn*10*)*2* (Tet^s)	Tet^s derivative of LC80
LC84	As LC82 plus *katE12*::Tn*10*	P1(UM120) × LC82
LC87	As MC4100ΔahpCF plus *zbe-279*::Tn*10*	P1(SK2255) × MC4100ΔahpCF
LC89	As GS022 plus Δ*ahpCF′ kan*::*′ahpF zbe-279*::Tn*10*	P1(LC87) × GS022

foxide (DMSO) was purchased from Fisher. Water for buffers was purified with a Labconco Water Pro PS system using deionized water as the feedstock.

Luria broth (LB) contained (per liter) 10 g of Bactotryptone (Difco), 5 g of yeast extract (Difco), and 10 g of sodium chloride. To prevent the photochemical formation of hydrogen peroxide, LB medium was shielded from light and used within 24 h of its preparation. Glucose minimal medium consisted of minimal A salts (19) with 1 mM $MgSO_4 \cdot 7H_2O$, 5 mg of thiamine, and 2 g of glucose per liter. However, to minimize the chemical production of hydrogen peroxide, the glucose medium used in some experiments (as noted) was prepared immediately before use and contained only 0.5 g of glucose per liter. Tetracycline, kanamycin, spectinomycin, and chloramphenicol were used at 12, 40, 120, and 20 μg/ml, respectively.

Growth conditions and strains. Cultures were routinely grown at 37°C. Aerobic cultures were grown in shaking flasks; anaerobic cultures were grown in a Coy chamber (Coy Laboratory Products, Inc.) under 85% N_2–10% H_2–5% CO_2. The optical densities (OD) of all cultures were measured at 600 nm.

The strains used in this study were derived from *E. coli* K-12 and are listed in Table 1; isogenic strains were used in all experiments. Mutations were introduced into strains via P1 transduction (19). To avoid the outgrowth of suppressed strains, the *katG, katE*, *oxyR*, and Tn*10*-linked *ahp* mutations were transduced and selected under anaerobic conditions. The presence of *katG* null mutations was confirmed by an *o*-dianisidine assay for hydroperoxidase I (HPI) activity (below). *katE* mutants failed to form bubbles on plates when colonies were overlaid with a drop of 30% H_2O_2. Mutants lacking *oxyR* were identified by their inability to induce HPI when anaerobic, exponentially growing cultures at 0.1 OD in LB medium were exposed to 60 μM H_2O_2 for 45 min. Mutations in *gshA* were confirmed by measurements of intracellular thiol levels using DTNB (13).

Alkyl hydroperoxide reductase cannot easily be assayed in extracts, because its subunits dissociate (11, 26). However, *ahpCF* mutants formed a large zone of growth inhibition when 10 μl of 3% cumene hydroperoxide was spotted onto a filter disk and laid onto a mutant-seeded plate (32). The excision of Tn*10* elements containing a tetracycline resistance marker was achieved at 42°C by standard methods (16).

H_2O_2 detection. In the presence of H_2O_2, horseradish peroxidase (HRP) oxidizes AR to the fluorescent product resorufin. One milligram of AR was dissolved in 0.78 ml of DMSO, and 0.75 ml of this solution was then diluted into 18 ml of 50 mM potassium phosphate (KPi, pH 7.8) to generate a 200 μM stock solution. This solution was shielded from light. HRP was dissolved in 50 mM KPi (pH 7.8) to 0.02 mg/ml. To measure H_2O_2, 0.45 ml of sample was mixed with 0.25 ml of AR and 0.25 ml of HRP. Fluorescence was then measured in a Shimadzu RF Mini-150 fluorometer and converted to H_2O_2 concentration using a curve obtained from standard samples. Note that a small amount of H_2O_2 is generated by the dye/HRP detection system itself; this amount was accounted for by the standard curve.

H_2O_2 scavenging by whole cells. Cultures were grown aerobically for at least four generations to 0.1 to 0.3 OD. Cells were pelleted in a microcentrifuge, washed twice, and resuspended in room temperature phosphate-buffered saline (PBS) at an OD of 0.1. H_2O_2 was added to the appropriate final concentration (see figure legends). At intervals, 0.45-ml aliquots were removed, diluted when necessary, and assayed immediately for H_2O_2 content by the AR/HRP method.

LB medium was used for most experiments. However, in experiments designed to measure the scavenging of trace H_2O_2, cultures were grown in minimal A glucose medium containing 0.5 mM each of the 20 L-amino acids. Cells were then washed and resuspended into fresh medium containing only 0.02% glucose and 0.05 mM amino acids, so that metabolism was active and could provide Ahp with reductants, yet the amount of H_2O_2 generated by the medium was minimal. When cyanide was included in the medium, fluorescence development was permitted to proceed for 10 min before a final reading was made, since the carryover of cyanide inhibits the activity of HRP.

Measurement of H_2O_2 accumulation in cell cultures. To detect H_2O_2 formation by drug-treated cells, we exposed log-phase cultures (0.2 OD) in LB medium to 300 μM plumbagin, an amount that is sufficient to consume 8 μM oxygen per min in a cyanide-resistant (nonrespiratory) fashion. After 16 min, cells were removed by centrifugation, and the residual H_2O_2 in the medium was assayed by the HRP/*o*-dianisidine assay (18).

H_2O_2 can be detected with greater sensitivity in defined medium using the

AR/HRP assay. Cells were grown anaerobically overnight in minimal A medium containing 0.2% glucose, diluted to 0.01 OD, and grown anaerobically for four generations. These log-phase cells were then subcultured to 0.05 OD in fresh aerobic minimal A medium containing 0.05% glucose. At 0.05 and 0.1 OD, the culture medium was assayed for H_2O_2. Hydrogen peroxide levels were also determined in sterile medium that was incubated at 37°C for an equivalent time. The low glucose concentration was used in order to minimize H_2O_2 production by salt-catalyzed glucose autooxidation (below).

H_2O_2 production rates. In order to monitor continuously the intracellular formation of H_2O_2, extracellular H_2O_2 levels were measured. Cells were first grown overnight in minimal A 0.2% glucose medium containing 0.5 mM each of the 20 amino acids. Most cultures were then diluted to ≈0.001 OD and grown aerobically to an OD of ≈0.1; however, JI377 was grown only to an OD of ≈0.05 in order to measure H_2O_2 production before growth was significantly inhibited. Cells were then washed in fresh medium containing only 0.02% glucose and 0.05 mM amino acids, resuspended at an OD of 0.1 in the same medium, and incubated with shaking at either 25 or 37°C. Glucose was added to the minimal A salts immediately before use. Aliquots were removed at intervals, and their H_2O_2 content was measured. The rate of H_2O_2 production was normalized to the cytoplasmic volume of the suspended cells, using a standard ratio of 0.47 μl of internal volume per 1 ml of 1.0 OD of *E. coli* (10).

Total catalase activity. Aerobic exponential-phase cells were harvested at 0.5 OD, washed in cold 50 mM KPi, resuspended in 1/4 the original volume, and lysed by passage through a French press. Extracts were centrifuged at 13,000 × *g* for 20 min to remove cell debris and then stored on ice. Catalase activity was measured in a 1-ml reaction mixture containing 50 μl of extract, 1 mM H_2O_2, and PBS (pH 7.3). At various time points, 10 μl was removed, diluted 1:500 in PBS, and assayed for H_2O_2 by the AR/HRP procedure.

HPI assay. Cultures were grown aerobically for five to six generations in LB medium. Chloramphenicol was added, and cultures were incubated for 5 min. Cells were then washed in PBS containing chloramphenicol, and cell pellets were frozen on dry ice. Within an hour the cells were resuspended in 1/10 the culture volume of 10 mM KPi buffer (pH 6.4) and lysed by sonication. Debris was centrifuged out at 13,000 × *g* for 20 min. HPI activity was assayed by the *o*-dianisidine method (18).

β-Galactosidase assay. Studies of *katG*::*lacZ* expression used a λRS45 ϕ(*katG*::*lacZ*) lysogen. Cultures were grown overnight under anaerobic conditions, diluted to an OD of 0.01 in LB, and grown anaerobically to an OD of 0.1. For measurement of anaerobic expression, chloramphenicol was added and cultures were harvested. For measurement of aerobic expression, cultures were shifted into air and grown with vigorous shaking to an OD of 0.3 to 0.4. Where indicated, 13,000 U of catalase was added every 15 min. Repeated additions were necessary because catalase rapidly loses activity in cell cultures.

At harvest, cultures were centrifuged. Pellets were washed in 50 mM cold KPi buffer (pH 7.0) with special concern to remove any residual exogenous catalase. Cells were resuspended in 50 mM KPi buffer (pH 7.0) at 1/10 the culture volume and lysed by French press. Cell debris was removed by centrifugation at 13,000 × *g* for 20 min. Extracts were incubated at 28°C for 5 min before being assayed. β-Galactosidase activity was assayed in a 1.2-ml reaction mixture consisting of 0.2 ml of ONPG (*o*-nitrophenyl-β-D-galactopyranoside, 4 mg/ml), extract, and Z-buffer (19) at 28°C. Change in absorption over time was monitored at 420 nm. Protein concentrations of all extracts were determined using a Coomassie dye-based assay by Pierce. All assays were performed on duplicate samples, and the values were then averaged.

Growth experiments. Care was taken to determine whether the poor aerobic growth of the Ahp^- Kat^- strain (JI377) was due to endogenous H_2O_2 or H_2O_2 that was chemically generated by autooxidation of the medium. Low-peroxide medium, which contains ≈0.02 μM H_2O_2, was prepared by adding filter-sterilized glucose to anaerobic minimal A salts immediately before inoculation. During aerobic incubation at 37°C, this medium accumulates ≈0.04 μM H_2O_2 per h. JI377 was inoculated from an anaerobic overnight culture to 0.01 OD in anaerobic, low-peroxide medium and grown anaerobically to 0.1 OD. Cells were then subcultured into the same medium and shifted into air. Growth was monitored during the first 2 h, during which the vast majority of H_2O_2 present in the medium was generated by the cells.

In some experiments, anaerobic MG1655 was mixed with JI377 at a 9:1 ratio in LB, the mixed culture was diluted to 0.01 OD in aerobic LB, and the growth of both strains was monitored by intermittent dilution and plating on LB plates (to quantify total viable cells) and LB plus tetracycline (to quantify viable JI377). In other experiments JI377 was shifted into aerobic LB in pure culture, and 6,500 U of catalase was added every 15 min. JI377 growth was monitored by plating, because the absorbance of catalase interferes with measurement of biomass by optical density.

Disk diffusion. Standing overnight cultures in LB medium were diluted to 0.005 OD in aerobic LB and then grown to an OD of ≈0.1. Cultures were diluted 1:10, and 100 μl was spread on LB plates. Round sterile filters (1-cm diameter) were placed in the center of the plates and spotted with 25 μl of 3% H_2O_2. Plates were incubated at 37°C overnight. The distance from the edge of the disk to the edge of the growth zone was measured. This experiment was performed in triplicate; mean values are reported.

RESULTS

Catalase is not the primary scavenger of low-level H_2O_2. Inside bacteria, antibiotics such as paraquat, plumbagin, and juglone are cyclically reduced by redox enzymes and oxidized by molecular oxygen, thereby generating superoxide and, upon its dismutation, hydrogen peroxide. The rate at which H_2O_2 is made inside drug-treated *E. coli* can be determined by measurement of nonrespiratory oxygen consumption in the presence of cyanide. However, we were unable to detect H_2O_2 accumulating in the medium of plumbagin-treated wild-type cells, despite the fact that oxygen consumption measurements indicated that the amount of H_2O_2 should have been easily within our detection limits (data not shown).

E. coli contains two catalases, and we anticipated that these were responsible for scavenging the H_2O_2. However, when the experiments were repeated with strain UM1, which has point mutations in both of the catalase structural genes (*katG* and *katE*), H_2O_2 was again undetectable. By adding 1.5 μM H_2O_2 to the bacterial culture, we found that UM1 degraded H_2O_2 as rapidly as did its wild-type parent (data not shown).

HPI, the catalase encoded by *katG*, exhibits weak NAD(P)H-dependent peroxidase activity in vitro, and the *katG17* mutation that is present in UM1 eliminates catalase but not peroxidase activity. Therefore, we wondered whether the residual peroxidase activity of HPI was responsible for the scavenging activity of UM1. True null mutations of both *katE* and *katG* were transduced into the wild-type strain MG1655, and the H_2O_2 scavenging measurements were repeated. This catalase double mutant had no detectable catalase or HPI peroxidase activity in vitro, but it still scavenged 1.5 μM H_2O_2 approximately as rapidly as its wild-type parent (Fig. 1). Therefore, *E. coli* must have another means of efficiently scavenging H_2O_2.

Ahp is the source of scavenging in a catalase null mutant. Other mechanisms of scavenging were considered. Some α-ketoacids, such as pyruvate, can be excreted into the medium of glucose-fed cells, and these can chemically reduce H_2O_2 in an oxidative decarboxylation reaction (4). In separate work we have observed that both the respiratory cytochrome *o* and cytochrome *d* oxidases have weak peroxidase activities (A. Nguyen and J. A. Imlay, submitted for publication). Finally, alkyl hydroperoxide reductase (Ahp) has been shown to accept H_2O_2 as a substrate in vitro (22).

Spent medium was examined, but it did not have significant scavenging activity. Cyanide competitively inhibits H_2O_2 binding by cytochrome oxidases but did not diminish scavenging by a *katG katE* mutant; the same result was obtained when *cyo* and *cyd* mutations were placed in this background. Thus, neither growth medium nor cytochrome oxidases provided the catalase-independent scavenging activity.

Ahp is an NAD(P)H-dependent peroxidase that rapidly reduces organic hydroperoxides as diverse as cumene and *t*-

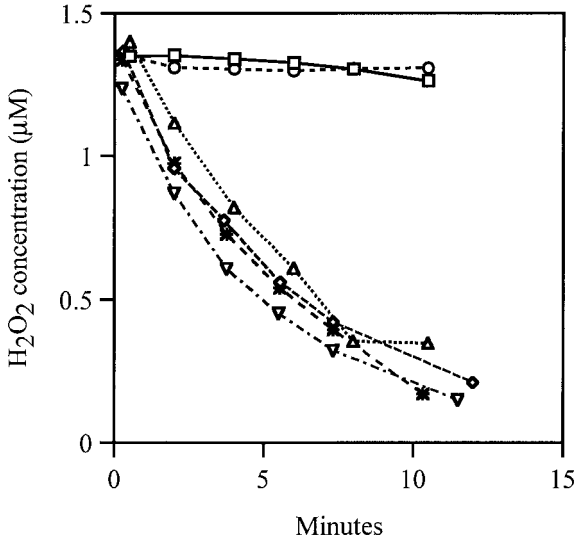

FIG. 1. Ahp scavenges H_2O_2 in a Kat$^-$ strain. Cultures of MG1655 (wild type, ▽), JI367 (*katG katE*, ◇), JI370 (*ahpCF*, *), JI377 (*ahpCF katG katE*, ○), JI374 (*ahpCF katG*, □), and JI372 (*ahpCF katE*, △) were grown aerobically in LB and resuspended in PBS at an OD of 0.1. H_2O_2 was added at a final concentration of 1.5 μM. At various time points after addition of H_2O_2, the H_2O_2 concentration was measured as described in Materials and Methods.

butylhydroperoxide (11). Niimura et al. subsequently found some activity with hydrogen peroxide as the substrate, although we are not aware of any comparative study of turnover numbers (22). Ahp, like HPI, is a member of the OxyR regulon in diverse bacteria (15, 20, 24). OxyR is activated by organic hydroperoxides as well as by H_2O_2, and it has become accepted that the physiological role of Ahp is to scavenge organic hydroperoxides. Mutants that lack Ahp are indeed hypersensitive to growth inhibition by organic hydroperoxides, which are not substrates for catalase (32).

A null mutant lacking Ahp scavenged H_2O_2 as well as did its wild-type parent (Fig. 1). However, in contrast to the *katG katE* strain, an *ahpCF katG katE* triple mutant exhibited virtually no scavenging activity. An *ahpCF katG* mutant also failed to scavenge H_2O_2, although an *ahpCF katE* mutant did so (Fig. 1). Thus, Ahp provides the scavenging activity that persists in catalase null mutants.

HPI induction can compensate for loss of Ahp. We wished to learn which enzyme, Ahp or HPI, was the predominant scavenger in wild-type cells. Because the *ahpCF* and *katG* single mutants each scavenged H_2O_2 at the same rate as did wild-type cells (Fig. 1), we inferred that one of the enzymes might be induced to compensate for the absence of the other. Both *ahpCF* and *katG* are positively regulated by the OxyR regulon, and a mutation in the constitutive scavenger might cause intracellular H_2O_2 to accumulate until OxyR activated the expression of the other, ultimately restoring wild-type levels of H_2O_2 scavenging.

We observed that an *ahpCF* mutant contained sevenfold more total catalase (including both HPI and HPII) than did wild-type cells. The *o*-dianisidine peroxidase activity, which specifically reflects the HPI titer, was increased 10-fold. Similarly, the β-galactosidase activity of an aerobic λRS45 *katG*::*lacZ* lysogen was increased 10-fold when the *ahpCF* null mutation was introduced, rising from 0.04 ± 0.01 to 0.39 ± 0.05 β-galactosidase U/mg. In contrast, the introduction of *katG* (LC80), *katE* (HDO3), or both *katG* and *katE* (LC84) mutations did not increase β-galactosidase activity at all (0.04 ± 0.01, 0.03 ± 0.01, and 0.04 ± 0.01 U/mg, respectively). These results agreed with the observations of Rosner and Storz (27) and Ritz et al. (25a).

TABLE 2. Endogenous H_2O_2 activates the OxyR regulon in an *ahpCF* mutant

Strain[a]	Mean β-galactosidase activity (U/mg of protein) ± SD		
	Anaerobic	Aerobic	Aerobic + catalase[b]
Ahp$^+$ OxyR$^+$ (GSO22)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Ahp$^-$ OxyR$^+$ (LC70)	0.03 ± 0.01	0.35 ± 0.03	0.18 ± 0.02
Ahp$^-$ OxyR$^-$ (LC74)	0.03 ± 0.01	0.03 ± 0.01	ND[c]

[a] Strains were isogenic and harbored λRS45 (*katG*::*lacZ*).
[b] Exogenous catalase (30 μl) was added before the culture was aerated and then every 15 min until it was iced.
[c] ND, not determined.

Induction of *katG* in the *ahpCF* mutant was blocked by an *oxyR* mutation (Table 2). To ensure that induction of the OxyR regulon was not due to a suppressor mutation secondary to the *ahpCF* mutation, the *ahpCF* null allele was retransduced into the *katG*::*lacZ* lysogen under anaerobic conditions. Once again, the fusion was induced when the transductant was cultured in aerobic medium (data not shown). These data demonstrate that in wild-type cells Ahp rather than catalase is the primary scavenger of an endogenous inducer of the OxyR regulon.

The induction of *katG*::*lacZ* in *ahp* cultures was partially averted by the addition to the growth medium of exogenous catalase (Table 2). Since catalase scavenges only H_2O_2, we infer that H_2O_2 is the species that accumulates in these cultures and activates the OxyR regulon. The extracellular catalase may have failed to completely block induction both because of its instability and because extracellular scavengers cannot fully eradicate intracellular H_2O_2 accumulation (30). Alternatively, it is possible that an additional inducer was present.

Ahp and HPI have discrete roles in scavenging H_2O_2. Mutants that contain only one or the other scavenger exhibited very different dose-response curves (Fig. 2). Whole cells that contained only Ahp scavenged low concentrations of H_2O_2 very effectively, exhibiting a half-maximal rate when the extracellular concentration of H_2O_2 was about 5 μM. The activity became saturated when extracellular H_2O_2 exceeded 20 μM (Fig. 2). In contrast, HPI-expressing cells were not saturated by even millimolar concentrations of H_2O_2, consistent with its K_m of 5.9 mM (9). The dose-response curves suggest that the primary role of HPI might be to scavenge higher concentrations of H_2O_2, against which Ahp is ineffective.

To directly contrast the kinetic behaviors of Ahp and HPI, we measured the rates at which cells decomposed low (0.1 μM) and high (150 μM) concentrations of H_2O_2. (To simplify interpretation, the strains used in these experiments lacked HPII. However, control experiments demonstrated that HPII

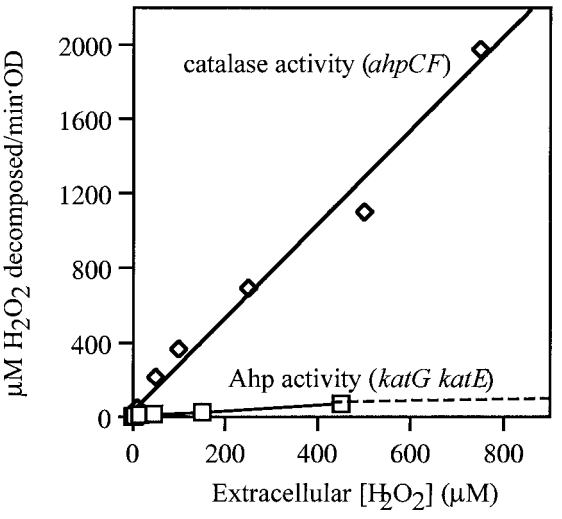

FIG. 2. Dependence of scavenging rate on H_2O_2 concentration. Rates of H_2O_2 decomposition were measured in dilute suspensions of JI370 (*ahpCF*, ◇) and JI367 (*katG katE*, □). Rates were normalized to a value of 1.0 OD.

provides significant scavenging activity only in stationary phase, when it is induced by RpoS [28].) An HPI^- Ahp^+ mutant scavenged 0.1 μM H_2O_2 slightly more rapidly than did an HPI^+ Ahp^- mutant, despite the fact that the latter strain has 10-fold-induced levels of HPI (Fig. 3, left panel). Thus, in wild-type *E. coli* most scavenging of low-dose H_2O_2 must be done by Ahp. Conversely, the HPI^- Ahp^+ strain was poor at degrading 150 μM H_2O_2, unlike the HPI^+ Ahp^- and HPI^+ Ahp^+ strains (Fig. 3, right panel). Although Ahp can be inactivated by H_2O_2 in vitro (11), its poor activity in vivo seemed not to stem from this problem, since normal scavenging activity was observed when the 150 μM H_2O_2 was washed away and the cells were exposed to 1.5 μM H_2O_2 (data not shown). The Ahp^- mutant scavenged 150 μM H_2O_2 twice as rapidly as did the wild type, because HPI^+ was induced (30).

TABLE 3. HPI (*katG*) is the primary scavenger of supranormal H_2O_2 concentrations[a]

Strain	Zone of inhibition[b] (cm)
Wild type	1.1
katG	1.6
ahpCF	1.1
katG katE	1.6
ahpCF katG	2.9
ahpCF katG katE	3.1

[a] Assay was done by disk diffusion of 25 μl of 3% (880 mM) H_2O_2. Assay was done in triplicate, and trends were always consistent. One data set is shown. Strains used were MG1655 (wild type), JI364 (*katG*), JI370 (*ahpCF*), JI367 (*katG katE*), JI374 (*ahpCF katG*), and JI377 (*ahpCF katG katE*).
[b] Distance of clearing from the edge of the disk.

These data indicate that Ahp and HPI have distinct roles: Ahp is more effective at scavenging very low concentrations of H_2O_2, while HPI is the more effective enzyme at higher concentrations. The dominance of HPI at supranormal levels of H_2O_2 was also apparent in disk diffusion assays, which test the ability of strains to degrade high concentrations of H_2O_2 to a level that permits growth. The *katG* mutant was hypersensitive, while the *ahpCF* mutant showed wild-type resistance (Table 3). The *ahpCF* mutation debilitated only strains that lacked HPI. As before, the addition of a *katE* mutation did not affect the sensitivity of any strain.

Calculation of endogenous H_2O_2 production during aerobic growth. Substantial H_2O_2 accumulated in the medium of Ahp^- Kat^- cultures. The extracellular concentration of H_2O_2 rose to

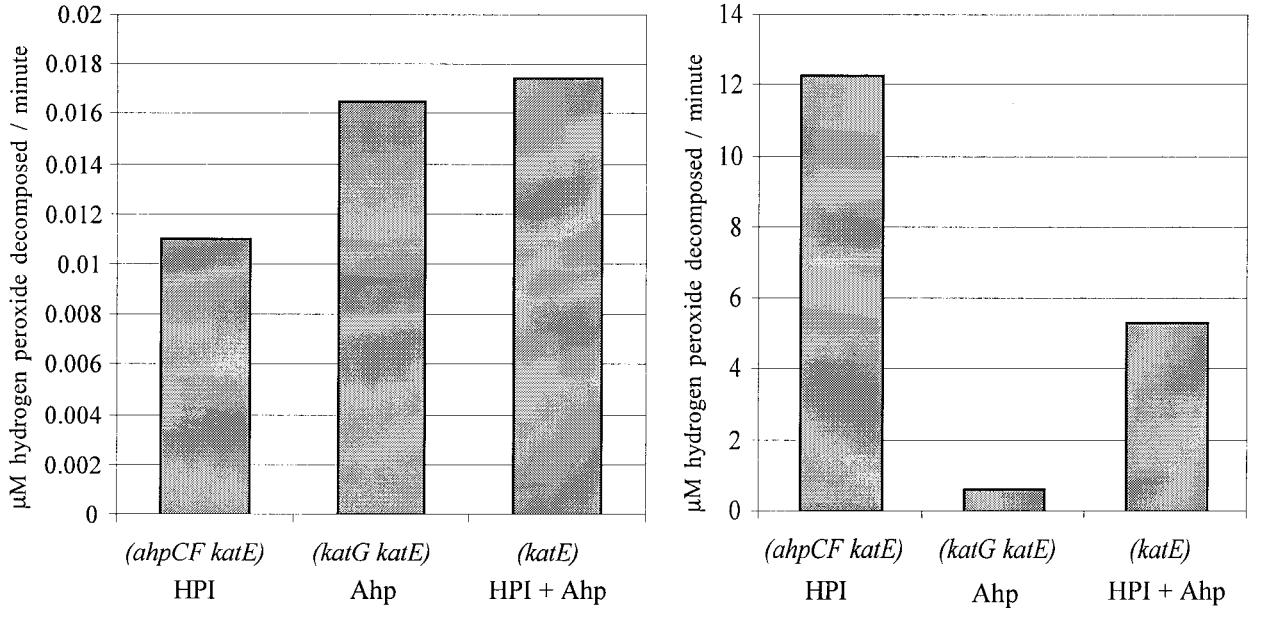

FIG. 3. Distinct efficiencies of Ahp and HPI at different H_2O_2 concentrations. H_2O_2 was added at a final concentration of 0.1 μM (right panel) and 150 μM (left panel) to cultures of JI372 (*ahpCF katE*), JI367 (*katG katE*), and JI362 (*katE*). Two minutes after addition of H_2O_2, the H_2O_2 concentration was measured as described in Materials and Methods.

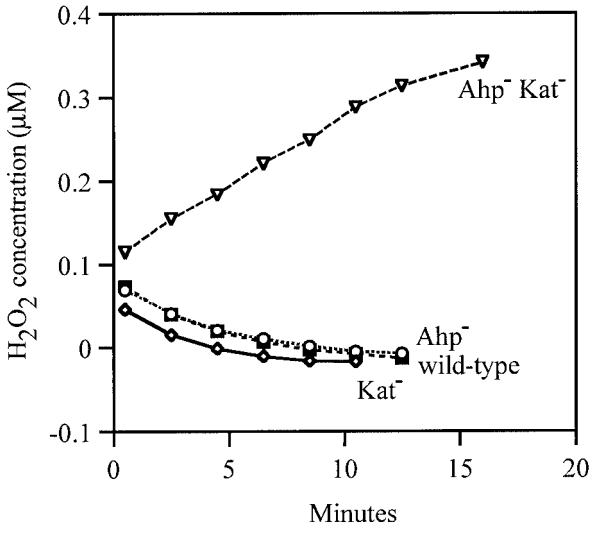

FIG. 4. H_2O_2 production by Ahp$^-$ Kat$^-$ cells. MG1655 (wild type), JI370 (*ahpCF*), JI367 (*katG katE*), and JI377 (*ahpCF katG katE*) were grown aerobically in LB and resuspended in 37°C minimal A salts containing 0.2% glucose. At various time points after resuspension, the H_2O_2 concentration of the medium was measured. (The H_2O_2 levels drop for the three scavenger-proficient strains because these strains degrade the 0.05 μM H_2O_2 that is present in the initial medium.)

1.8 μM during aerobic growth in minimal 0.05% glucose medium when cells were cultured for a single generation, from 0.05 to 0.10 OD_{600}. In contrast, the H_2O_2 concentrations of wild-type (MG1655), Kat$^-$ (JI367), and Ahp$^-$ (JI370) cultures were below our detection limit of ≈0.04 μM H_2O_2. The fact that the H_2O_2 concentration was so low in Ahp$^-$ cultures was initially surprising, given that sufficient H_2O_2 was present to activate the OxyR protein. However, subsequent work indicated that the concentration of H_2O_2 inside these cells may have been substantially higher than that outside them (30).

Kat$^-$ Ahp$^-$ strains have <5% of the scavenging activity of wild-type cells, so that virtually all of the H_2O_2 that enters or is formed within these cells diffuses out without being scavenged (30). For this reason the measurement of excreted H_2O_2 is a valid proxy for measurement of endogenous H_2O_2 formation. Using this strain, we quantitated the rate at which *E. coli* generates H_2O_2 (Fig. 4). Measurements were made after exponentially growing cells were suspended in fresh glucose/amino acids medium at 37°C (and do not necessarily apply in other media). The rate of H_2O_2 formation, normalized to cell volume, was 14 μM H_2O_2/s at 37°C. This result is consistent with earlier predictions (Discussion).

Inability to scavenge endogenous H_2O_2 causes a growth defect. It has long been suspected that aerobic metabolism generates enough H_2O_2 to damage cells that cannot scavenge it. No growth defects were apparent in the *ahpCF* mutant; we presume this is because the cell compensates sufficiently for the loss of *ahpCF* by inducing *katG*. However, the Ahp$^-$ Kat$^-$ strain grew poorly in all aerobic media that were tested (Fig. 5). Growth slowed progressively over time, and when cells were repeatedly subcultured, growth often stopped entirely (Fig. 5B). This may reflect the continual accumulation of damage in the cell. Growth was particularly poor on defined media that lacked amino acids (data not shown). Wild-type growth was restored when catalase was added to the medium (Fig. 5C) or when the mutant was cultured anaerobically (not shown).

Special efforts were undertaken to confirm that the growth defect was due to endogenous H_2O_2 rather than H_2O_2 made by autooxidation of the glucose medium. Cells were cultured anaerobically in glucose medium to log phase and then diluted into fresh aerobic "peroxide-free" medium (Fig. 5D). Within the first half-hour the Ahp$^-$ Kat$^-$ strain grew more poorly than did its wild-type parent. Our measurements over this period confirmed that the H_2O_2 found in this culture was generated primarily by the cells rather than by glucose oxidation. Thus, aerobic *E. coli* generates enough H_2O_2 to debilitate the cell. In previous studies of catalase mutants, the toxicity of endogenous H_2O_2 was obscured by the scavenging action of Ahp.

The necessity for care in these experiments was underscored by the observation that medium that had been stored on the bench often contained micromolar amounts of H_2O_2, and dilution of even wild-type cells into stored medium was sufficient to transiently induce the OxyR regulon (data not shown).

DISCUSSION

H_2O_2 may be the sole physiological substrate of Ahp. The results of this study indicate that in exponentially growing *E. coli* Ahp is responsible for the degradation of low concentrations of hydrogen peroxide. Previously, Ahp was primarily associated with the detoxification of organic hydroperoxides. However, the range of organic peroxides that are good substrates for the enzyme indicates that its active site can accommodate virtually any ROOH, so it is not surprising that it reacts with HOOH as well. Earlier genetic studies confirmed that Ahp provides cellular resistance to organic hydroperoxides but found little role in resistance to H_2O_2. This result is now understandable: because catalases cannot degrade organic hydroperoxides, Ahp is likely to be the only enzyme in *E. coli* with that catalytic ability. At the same time, the role of Ahp in H_2O_2 decomposition was obscured because the widely employed disk inhibition assays confront cells with high H_2O_2 concentrations that are more efficiently decomposed by catalases.

It is not clear whether *E. coli* must ever detoxify organic hydroperoxides in nature. Lipid hydroperoxides were suggested to be the physiological substrates for Ahp, but *E. coli* lacks the polyunsaturated fatty acids that appear to be necessary for lipid peroxidation (1), and we are not aware of any study that has documented the recovery of peroxidized lipids from this bacterium. It is possible that organic hydroperoxides are pseudosubstrates for an enzyme whose only role in nature is the decomposition of hydrogen peroxide.

Why does *E. coli* use multiple scavengers? In general, all peroxidases will be inferior to catalases at scavenging high concentrations of H_2O_2 because peroxidases can turn over only as quickly as the cell can provide a reductant to them. Catalases escape this restriction. For example, the HPI catalase of Ahp$^-$ *E. coli* degraded 500 μM H_2O_2 at a rate of 7×10^7 molecules cell^{-1} s^{-1}. To achieve the same rate using Ahp would require an equivalent amount of NADH, which substantially exceeds the rate at which metabolism generates NADH. (Glucose-saturated cells can generate enough NADH to respire 6×10^6 molecules of oxygen cell^{-1} s^{-1}.) The disparity is

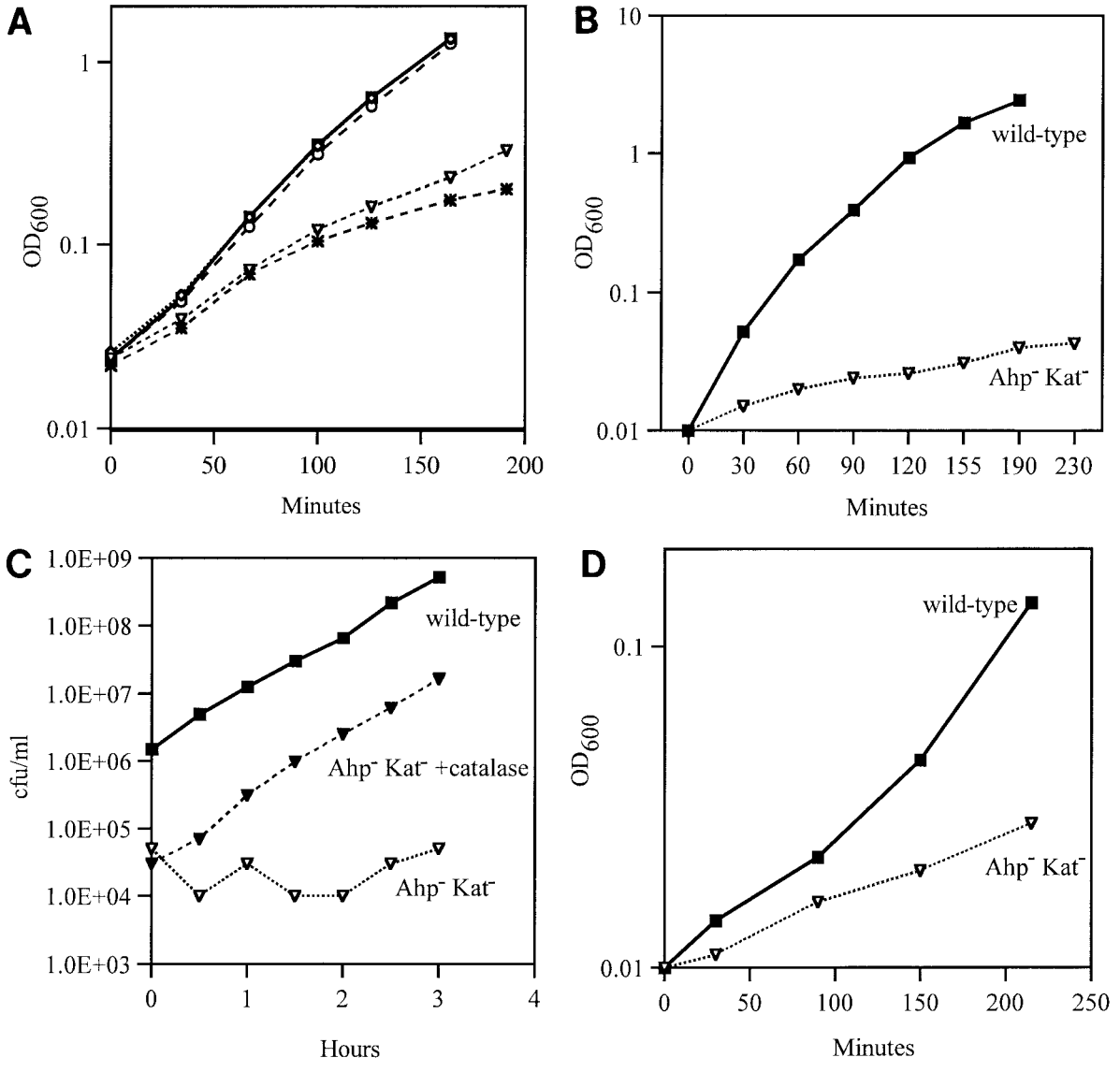

FIG. 5. An Ahp$^-$ Kat$^-$ strain has an aerobic growth defect. (A) Growth of MG1655 (wild type, ■), JI370 (*ahpCF*, ○), JI367 (*katG katE*, ◇), JI374 (*katG ahpCF*, *), and JI377 (*katG katE ahpCF*, ▽) in aerobic LB medium. (B) MG1655 and JI377 were grown aerobically in LB from 0.001 OD to mid-log phase, as for panel A. Cells were then subcultured into fresh LB at an OD of 0.01, and residual growth was observed. (C) Exogenous catalase protects against an aerobic growth defect in LB. MG1655 and JI377 were grown aerobically in fresh LB. Cultures were then subcultured to 0.01 OD in LB. Exogenous catalase was added to one culture of JI377 every 15 min to maintain catalase activity. At various time points, aliquots were removed from each culture and plated in selective top agar. Growth rate was determined the next day. (D) Endogenous H_2O_2 can be toxic to cells. Exponential anaerobic MG1655 and JI377 were subcultured into fresh aerobic peroxide-free minimal A glucose (0.2%) medium, and growth was monitored.

even greater when one considers decomposition of higher concentrations of H_2O_2. Therefore, catalases allow cells to degrade high concentrations of H_2O_2 far more quickly than would peroxidases alone.

The preference for Ahp at low H_2O_2 concentrations may derive from its greater catalytic efficiency. Since normal intracellular concentrations of H_2O_2 are well below the K_m of both enzymes, k_{cat}/K_m is the relevant kinetic parameter. The k_{cat}/K_m of the catalase activity of HPI is 9×10^5 M^{-1} s^{-1} (9); that of Ahp has not been reported, but the data of Niimura et al. (22) imply that it is at least 8×10^6 M^{-1} s^{-1}. The implication is that at least 10-fold more molecules of HPI need to be synthesized to provide the scavenging activity provided by Ahp. A second disadvantage to catalase may stem from the fact that enzymes that require reactions with two molecules of substrate to complete a catalytic cycle can have difficulty at low substrate concentrations, when an intermediate state is long-lived. Compound I, the divalently oxidized catalase intermediate, can be unstable and in some circumstances may reversibly deactivate (9), which would further diminish the ability of catalase to scavenge trace amounts of H_2O_2. Peroxidases may comprise an evolutionary solution to this problem.

The kinetic efficiency of HPII ($k_{cat}/K_m = 1.3 \times 10^6$ M^{-1} s^{-1}) (23), the stationary-phase catalase, is similar to that of HPI. It

seems reasonable that *E. coli* would increase its catalase titer in stationary phase, when there may not be sufficient NADH for Ahp to remain an effective scavenger. Gonzalez-Flecha and Demple reported that both catalases are induced as growth slows (8). It is not clear why scavenging efficiency could not be maintained by induction of HPI alone. For now, the benefit to *E. coli* of having two catalases instead of one remains obscure.

The compensatory interactions that we observed between catalase and Ahp synthesis have been observed previously in a wide range of bacteria. In *Xanthomonas campestris, Bacteroides fragilis,* and *Pseudomonas aeruginosa*, mutations in *ahp* cause catalase induction (20, 24, 26); this may be true in *Bacillus subtilis* as well (2). Conversely, *katG* mutations in *Mycobacterium tuberculosis* select for promoter upmutations in *ahp* (31). It seems likely that the division of labor found in *E. coli*, wherein Ahp scavenges low levels of H_2O_2 and catalase scavenges high levels, is widespread.

Mechanisms of H_2O_2 formation and cell damage. The existence of a strain unable to scavenge the H_2O_2 it produces has enabled us to measure the rate at which H_2O_2 is formed. In earlier studies in vitro, we determined that the respiratory chain was likely to be the primary source of H_2O_2 during aerobic growth on glucose, largely because of the autooxidizability of the NADH dehydrogenase II (18). This enzyme adventitiously transfers electrons to oxygen from its reduced flavin in a direct second-order chemical reaction. Other flavoenzymes can do so as well, and in circumstances where these other enzymes are especially abundant, they may be the predominant sources of superoxide and H_2O_2.

The rate at which H_2O_2 is likely to be formed in vivo can be predicted from the in vitro data. We found that 9 molecules of H_2O_2 were formed per 10,000 electrons flowing through the NADH dehydrogenase II (18). We do not know the fraction of the respiratory flux that flows through this enzyme during growth on glucose, but given that exponentially growing cells consume about 3.2 mM O_2/s, one extrapolates that the rate of H_2O_2 formation could be up to 12 μM/s. This is close to the rate determined in vivo in this study (14 μM/s). Efforts to test the sources of H_2O_2 production in vivo are under way.

Gonzalez-Flecha and Demple inferred rates of H_2O_2 production by intact, wild-type cells (ca. 1 to 2 μM/s) that are lower than those that we have reported (8). However, we observed rates similar to theirs when we suspended cells in room temperature buffer, as they did (3 μM/s). It is not surprising that H_2O_2 is most rapidly formed when metabolism is active and temperatures are high enough to overcome the activation energy (18). There was a procedural difference, however. Their calculation was based on measurements of steady-state concentrations of extracellular H_2O_2 in suspensions of cells: by assuming that catalase was the predominant scavenger of H_2O_2, they used measurements of its activity to calculate the rate of H_2O_2 production. In our experiments we found that H_2O_2 did not accumulate extracellularly due to the action of Ahp. These differences may reflect the different conditions under which the experiments were performed.

Inside growing cells, the steady-state concentration of H_2O_2 depends on the rates of its formation and of its dissipation. In this study we quantified the rate at which H_2O_2 is formed when *E. coli* is cultured under a particular set of growth conditions. In the accompanying work (30), we measured the processes that consume H_2O_2 and estimated the internal H_2O_2 concentration.

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