

Homeostatic Regulation of Intracellular Hydrogen Peroxide Concentration in Aerobically Growing *Escherichia coli*

BEATRIZ GONZÁLEZ-FLECHA AND BRUCE DEMPLE*

Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, Massachusetts 02115

Received 22 July 1996/Accepted 1 November 1996

The exponential phase of aerobic growth is associated with risk of endogenous oxidative stress in which cells need to cope with an ~10-fold increase in the rate of H₂O₂ generation. We addressed this issue by studying the regulation of the intracellular concentration of H₂O₂ in aerobically growing *Escherichia coli*. Intracellular H₂O₂ was kept at an almost constant steady-state value of ~0.2 μM (variation, less than twofold) over a broad range of cell densities in rich medium. This regulation was achieved in part by a transient increase in the OxyR-dependent transcription of the catalase gene *katG* (monitored by using a *katG::lacZ* operon fusion) during exponential growth, directly correlated with the increased rate of H₂O₂ generation. The OxyR-regulated alkyl hydroperoxide reductase encoded by *ahpFC* did not detectably affect H₂O₂ or catalase activity levels. Induction of *katG*, *ahpFC*, and perhaps other genes prevented the accumulation of oxidatively modified lipids but may not have protected DNA: the spontaneous mutation rate was significantly increased in both wild-type and Δ*oxyR* strains during exponential growth compared to that in these strains during lag or stationary phases. Strains lacking *oxyR* showed throughout growth an 8- to 10-fold-higher frequency of spontaneous mutation than was seen for wild-type bacteria. The *ahpΔ5* allele also had a mutator effect half of that of Δ*oxyR* in exponential and stationary phases and equal to that of Δ*oxyR* in lag phase, perhaps by affecting organic peroxide levels. These results show that *oxyR*-regulated catalase expression is not solely an emergency response of *E. coli* to environmental oxidative stress, but also that it mediates a homeostatic regulation of the H₂O₂ produced by normal aerobic metabolism. The activation of the *oxyR* regulon in this process occurs at much lower levels of H₂O₂ (~10⁻⁷ M) than those reported for *oxyR* activation by exogenous H₂O₂ (~10⁻⁵ M).

The intracellular concentration of hydrogen peroxide (H₂O₂) depends on the rates of H₂O₂ production and consumption. Physiological production of superoxide anion (O₂⁻) and the resulting H₂O₂ in *Escherichia coli* is mainly related to respiratory activity (13, 18) and varies considerably along the bacterial growth curve (13). Detoxification of H₂O₂ in *E. coli* is due mainly to two distinct species of catalase. The *katG* gene encodes a bifunctional catalase hydroperoxidase I (HP-I) (6). HP-I is active as a tetramer of 81-kDa subunits (6) and is transcriptionally induced by OxyR as a part of a genetic response to H₂O₂ (35). The *katE* gene codes for the monofunctional HP-II (25). HP-II is a tetramer of 78-kDa subunits (7) and is under the control of *rpoS*, which is activated in stationary-phase cells or upon various types of starvation (33). Recent studies have also shown an OxyR-independent regulation of HP-I by *rpoS* in stationary-phase cultures (19). The total catalase activity in *E. coli* changes along the growth curve (16) as a consequence of independent changes in HP-I and HP-II activity (26), but the level at which these changes are regulated has not been assessed.

OxyR also controls the expression of seven or eight other H₂O₂-inducible proteins in *Salmonella typhimurium* and *E. coli* (5, 15). *S. typhimurium* and *E. coli* Δ*oxyR* mutants are hypersensitive to H₂O₂ and other oxidative agents (5) and exhibit an elevated rate of spontaneous mutations during aerobic growth (15, 34). Increasing the expression of *katG* in Δ*oxyR* strains decreases the spontaneous mutation rates to near-wild-type levels (15, 34), which indicates that an increase in metabolically generated H₂O₂ is responsible for the elevated mutation rate.

However, the molecular mechanisms that limit H₂O₂ toxicity in normal cells are unclear. Specifically, the possibility that the Δ*oxyR* mutator effect applies only to rare individual cells with dramatically increased H₂O₂ levels rather than to the population as a whole remains. For example, elevated H₂O₂ levels in individual cells could arise from damage to components of the respiratory chain (13).

To define the basic features that affect H₂O₂ levels in *E. coli*, we have previously characterized the physiological sources of H₂O₂ production in wild-type *E. coli* (13). This previous study showed that significant amounts of hydrogen peroxide are produced during normal aerobic metabolism. In the present work, we have investigated the contribution of catalases and alkylhydroperoxidases to maintaining intracellular hydrogen peroxide at levels that are not toxic to aerobically growing cells. In addition, by measuring the amount of oxidative modifications to different cellular macromolecules, we have determined the extent of oxidative damage that occurs during normal aerobic metabolism.

MATERIALS AND METHODS

Reagents. Ampicillin, glucose, tetracycline, scopoletin, thiobarbituric acid, rifampin, bovine serum albumin, 2,4-dinitrophenylhydrazine (DNPH), trichloroacetic acid (TCA), guanidine hydrochloride, streptomycin sulfate, horseradish peroxidase type VI, and bovine catalase were purchased from Sigma Chemical Co. All other reagents used in this study were analytical grade.

Bacterial strains and growth conditions. Table 1 lists the strains and plasmids used in these studies. Strain BGF1033 was constructed by transduction (29) of the *rpoS13::Tn10* allele from strain UM257 into strain BGF933. Strains BGF611 and BGF711 were constructed by transduction of *katG17::Tn10* and *katE12::Tn10* from UM202 and UM254, respectively, into AB1157. The genotype of *rpoS* strains was confirmed by the H₂O₂-bubbling spot test described by Huisman and Kolter (17).

Cells were inoculated into Luria-Bertani medium (LB) broth (29) containing the appropriate antibiotic and incubated overnight at 37°C with gentle shaking (100 rpm). For experimental measurements, the saturated cultures were diluted 100-fold into fresh LB broth and incubated at 37°C for the times indicated. The

* Corresponding author. Mailing address: Department of Molecular and Cellular Toxicology, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115. E-mail: demple@mbcrr.harvard.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic	Source or reference
Strains		
AB1157	F ⁻ <i>thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx-33</i>	Laboratory stock
BGF611	As AB1157, but <i>katG17::Tn10</i>	This work
BGF711	As AB1157, but <i>katE12::Tn10</i>	This work
BGF931	As RK4936, but $\lambda[\Phi(katG':lacZ)]$	13
BGF933	As TA4112, but $\lambda[\Phi(katG':lacZ)]$	13
BGF1033	As BGF933, but <i>rpoS13::Tn10</i>	This work
BGF1041	As AB1157, but <i>rpoS13::Tn10</i> $\lambda[\Phi(katG':lacZ)]$	This work
MC4100	$\Delta(lac)U169 rpsL$	Laboratory stock
MP180	HfrH <i>thi</i>	32
RK4936	<i>araD139 (argF-lac)205 glbB5301 non-(gyrA219)relA1 rpsL150 metE70 btuB::Tn10</i>	Laboratory stock
RR1	<i>pro leu rpsL hsdM hsdR endI lacY</i>	2
TA4112	As RK4936, but $\Delta(oxyR btuB)3$	5
TA4482	As RK4936, but <i>ahpD5 zij-602::Tn10</i>	37
UM202	As MP180, but <i>katG17::Tn10</i>	26
UM257	As RR1, but <i>rpoS13::Tn10 katG15 thyA</i>	30
UM254	As RR1, but <i>katE12::Tn10 katG15 thyA</i>	30
Plasmids		
pAT153	Vector Ap ^r Tc ^r	39
pUC18	Vector Ap ^r	40
pBT22	pAT153 containing <i>katG</i>	38
pKT181	pUC18 containing <i>oxyR</i>	36

following antibiotics were used at the concentrations indicated (in micrograms per milliliter): ampicillin, 100; tetracycline, 12.5; streptomycin, 50.

Hydrogen peroxide measurements and calculations. Intracellular concentrations of H₂O₂ were measured by the procedures previously described (12). Briefly, bacterial cultures taken at the indicated times were pelleted and resuspended at 10⁶ cells/ml in phosphate-buffered saline. H₂O₂ generated within cells passes through the membranes and equilibrates with the buffer. Samples of the cell suspensions were removed at various times and, after a brief centrifugation (1 min at 6,000 × g), H₂O₂ concentration in the supernatant was measured by a horseradish peroxidase-scopoletin assay (13). Complete equilibration of the intra- and extracellular H₂O₂ level occurs within 10 min in this assay (13).

The rate of H₂O₂ production was calculated from the experimental values of intracellular H₂O₂ and catalase concentration by using the steady-state assumption that the rate of H₂O₂ production equals the rate of H₂O₂ elimination. The rate of H₂O₂ consumption could be calculated because catalase activity is the major activity eliminating H₂O₂ in *E. coli* (24). We have used a simplified equation for describing catalase kinetics developed by Chance et al. (3): $d[H_2O_2]/dt = 2k_1 \cdot [Cat] \cdot [H_2O_2]$, where k_1 is the second-order rate constant for the utilization of H₂O₂ by catalase (k_1 for bacterial catalase = $0.5 \times 10^6 M^{-1} s^{-1}$, as calculated from the data of Claiborne and Fridovich [6]), $[H_2O_2]$ is the measured intracellular concentration of hydrogen peroxide determined as described above, and $[Cat]$ is the measured concentration of catalase. For these estimates, catalase protein concentration per milligram of total protein was calculated from the experimental pseudo-first-order reaction constant ($k' = k_1 \cdot [Cat]$) for the decrease in H₂O₂ absorption at 240 nm (3) with k_1 as described above. Catalase concentrations were estimated using a cellular volume of 3.2×10^{-15} liter (18).

Mutation frequency measurements. Strains to be scored for rifampin resistance (Rif^r) were grown for 1 h (lag phase), 4 h (exponential phase), and 16 h (stationary phase). A 1-ml aliquot of each culture was plated on an LB plate containing rifampin (125 µg/ml) in 3 ml of molten agar. Rif^r colonies were scored after 24 h of incubation at 37°C (15). Results were normalized to the number of cells plated (determined by plating serial dilutions on LB agar) to compare mutation frequencies among the different growth phases. Near anaerobiosis during the growth of strains in rifampin plates was achieved by placing them in a gas-tight Atmos bag (Aldrich) that was flushed and filled with pure argon gas.

Enzymatic activities. Cellular catalase concentrations and glutathione reductase activities were determined in cell lysates as described previously (12, 27) and normalized to the protein content of the lysate as measured by the method of Lowry et al. (28) with bovine serum albumin as the standard. One unit of catalase destroys 1 µmol of H₂O₂ per min. β-Galactosidase activity in sodium dodecyl

sulfate-CH₃Cl-treated cells was determined and normalized to the cell density as described by Miller (29). Absorbance measurements were carried out in a Perkin Elmer UV/Vis spectrophotometer (model Lambda 3A; Perkin Elmer, Oak Brook, Ill.).

Determination of carbonyl content. The content of carbonyl groups in oxidatively modified proteins was measured by determining the amount of 2,4-dinitrophenylhydrazone formed upon reaction with DNPH (8). After precipitation of nucleic acids with 1% streptomycin sulfate, samples (>1 mg of protein per ml) were treated with 2 mM DNPH at room temperature, usually for 1 h. In some experiments, a 15-min derivatization time was used, which gave results indistinguishable from those obtained with the longer incubation (14). Proteins were precipitated with 10% TCA, washed with ethanol-ethyl acetate (1:1), and redissolved in 6 M guanidine hydrochloride–20 mM potassium phosphate (pH 2.3) (23). Carbonyl content was calculated from the absorbance maximum of 2,4-dinitrophenylhydrazone at 390 nm normalized to the absorbance at 350 nm, with an $\epsilon_{390-350}$ of $22 mM^{-1} cm^{-1}$ (21). Results were expressed in nanomoles of carbonyl groups per milligram of protein.

Determination of TBARS. Thiobarbituric acid-reactive substances (TBARS) were measured in isolated membranes prepared by a standard procedure (18) from lag-, exponential-, and stationary-phase cultures. Cell suspensions were precipitated with TCA, centrifuged, and incubated with thiobarbituric acid for 10 min at 100°C in the presence of 10 mM butylhydroxytoluene (9). The amount of TBARS formed was expressed in nanomoles per milligram of protein, with an ϵ_{535} of $156 mM^{-1} cm^{-1}$ (1).

Statistics. Each result is indicated as the mean value of at least four independent experiments ± the standard error of the mean (SEM).

RESULTS

Regulation of the intracellular concentration of H₂O₂ in *E. coli*. Our previous studies (13) showed that the rate of H₂O₂ production increases >10-fold during exponential growth. We therefore measured the concentration of H₂O₂ in intact *E. coli* AB1157 as a function of aerobic growth in rich medium. These measurements revealed that the intracellular H₂O₂ concentration ranged from 0.13 to 0.25 µM (Fig. 1A), which points to the existence of regulatory systems that maintain intracellular levels of H₂O₂ within a narrow range. During the first 2 h of growth, a 50% decrease in the catalase activity was accompanied by a significant rise in the intracellular H₂O₂ concentration (Fig. 1A). The H₂O₂ level remained elevated for an additional 2 h of growth, after which the oxidative imbalance was counteracted by a 10-fold increase in the total catalase concentration to restore a lower H₂O₂ concentration (Fig. 1A).

To determine which enzyme was responsible for the increase in catalase content in mid- to late-exponential phase, we measured the catalase profile in strains lacking either HP-I (*katG* [BGF611]) or HP-II (*katE* [BGF711]). The total catalase activity increased only twofold during exponential phase (up to 6 h of growth) in the HP-I-deficient strain, whereas the pattern followed by the HP-II-deficient strain was similar to that of wild-type *E. coli*, but with slightly lower absolute values (Fig. 1B and C).

To test if HP-I was responsible for maintaining H₂O₂ levels, we measured this metabolite in the mutant strains. The intracellular concentration of H₂O₂ in BGF611 (*katG* mutant) was significantly increased compared to those in the *katG*⁺ strains at every time point except in the stationary phase. Thus, *katG*-encoded HP-I enzyme activity is a major activity that modulates H₂O₂ levels during exponential growth. However, the increase in the H₂O₂ level in BGF611 is less than might be expected for the mere loss of HP-I, which could indicate that there are compensating changes in these cells (see below). The HP-II enzyme made only a small contribution to the total catalase activity at 5 to 7 h of growth, but at 16 h it represented the dominant form after HP-I levels had decreased about threefold (Fig. 1C).

Since *katG* is dually regulated by *oxyR* and *rpoS*, we determined which of these regulators governs the exponential-phase increase in catalase. These studies employed a single-copy *katG::lacZ* operon fusion to report the transcriptional activity

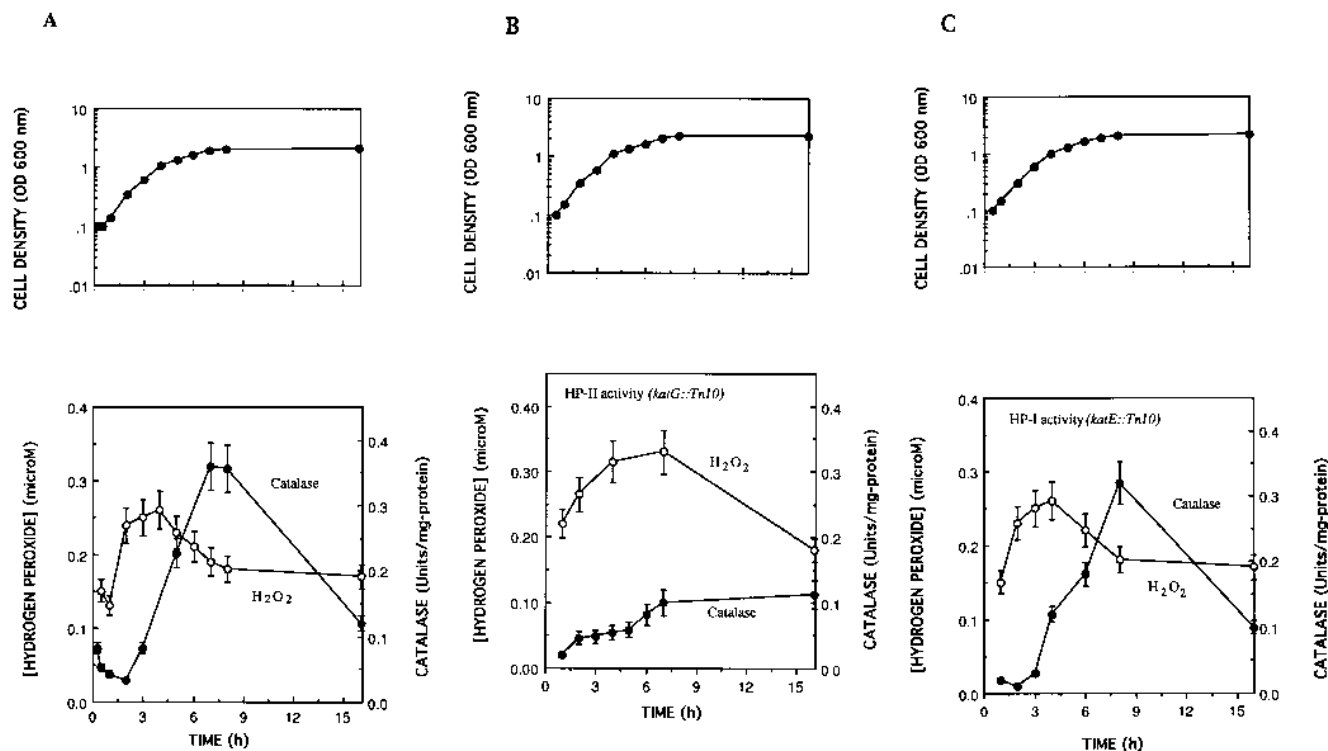


FIG. 1. Intracellular concentration of hydrogen peroxide and catalase during aerobic growth. Overnight cultures of *E. coli* AB1157 (wild type) (A), BGF611 (*katG* mutant) (B), or BGF711 (*katE* mutant) (C) were diluted 1/100 in fresh LB and incubated at 37°C with gentle shaking (100 rpm). At the indicated times, samples were taken to assay catalase activity (filled circles) and H₂O₂ concentration (open circles). The upper panel in each part of the figure shows bacterial growth monitored as optical density (OD) at 600 nm.

of *katG* in strains bearing the regulatory mutations. In *oxyR*⁺ *rpoS*⁺ cells, expression of the fusion (Fig. 2) essentially paralleled the variation in the catalase activity measured directly (Fig. 1C) ($R = 0.91$), suggesting that transcriptional regulation is the main or even the sole mechanism for growth-dependent catalase HP-I regulation. This transcriptional up-regulation was preserved in an *rpoS* mutant, greatly attenuated in an *oxyR* mutant, and completely absent from an *oxyR rpoS* double mutant (Fig. 2). These data suggested that OxyR activity varies continuously during growth, and this hypothesis was tested by monitoring the activity of another *oxyR*-regulated enzyme, glu-

tathione reductase. The pattern for this enzyme (Fig. 3) essentially paralleled that for HP-I catalase (Fig. 1C).

The *oxyR*-dependent changes in *katG* expression during growth could be related to either the increased rate of H₂O₂ production or to the steady-state level of H₂O₂. We therefore attempted to correlate these parameters with *katG* expression or with catalase activity. The rate of H₂O₂ production in strain BGF931 was linearly related to the expression of *katG*::*lacZ* (Fig. 4A) and to the catalase concentration (14). The intracellular concentration of H₂O₂ did not correlate well with either *katG*::*lacZ* expression (14) or catalase activity (Fig. 4B). We did not attempt to correlate H₂O₂ production and catalase

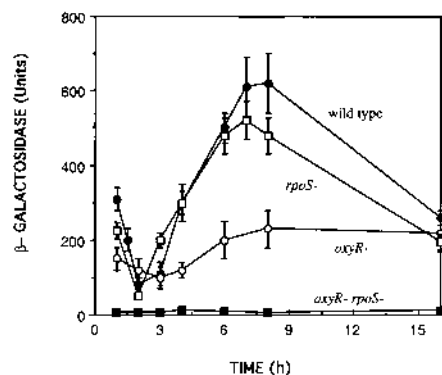


FIG. 2. Induction of a *katG*::*lacZ* operon fusion in aerobically growing *E. coli*. Cultures of strains BGF931 (wild type [filled circles]), BGF933 (Δ *oxyR* [open circles]), BGF1033 (Δ *oxyR rpoS* mutant [filled squares]), and BGF1041 (*rpoS* mutant [open squares]) were grown at 37°C in LB. Samples were taken every hour to assay β -galactosidase activity. Error bars show SEMs.

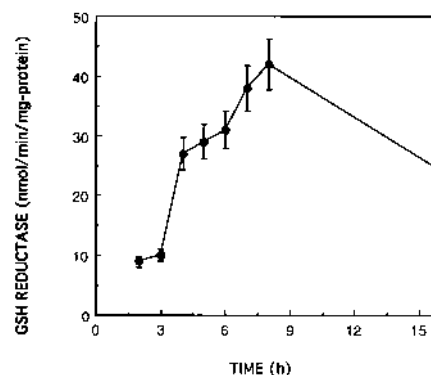


FIG. 3. Glutathione (GSH) reductase activity during aerobic growth of *E. coli* AB1157. Error bars show SEMs.

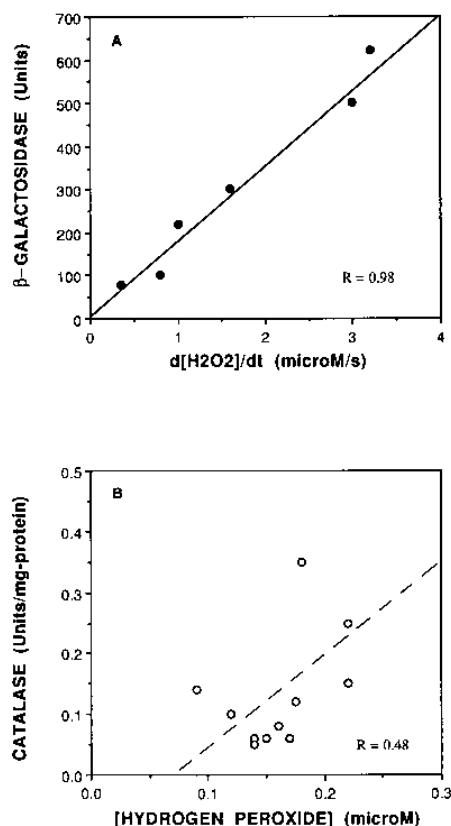


FIG. 4. β -Galactosidase activity from a *katG'::lacZ* operon fusion as a function of the rate of H₂O₂ generation (A) and catalase activity as a function of the intracellular concentration of H₂O₂ (B) during 2 to 16 h of growth.

levels, because these are not independent variables (Materials and Methods).

The effect of regulatory defects in *oxyR* or *rpoS* on the oxidative status of the cell was also examined. A $\Delta oxyR$ strain failed to increase catalase activity in lag and exponential phases, and total catalase remained depressed relative to that of wild-type cells, even in stationary phase (Fig. 5). As a result, the intracellular H₂O₂ concentrations in the $\Delta oxyR$ strain were increased to values ranging from 0.5 to 1 μ M (Fig. 5), much higher than those found for the *katG* mutant (Fig. 1B). Inter-

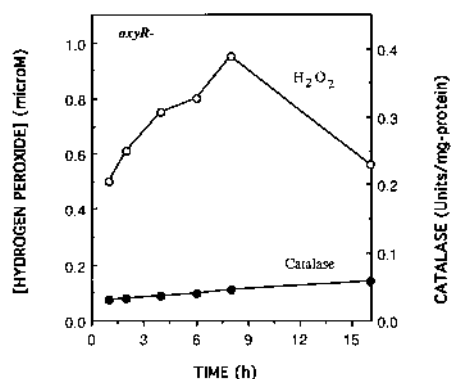


FIG. 5. Intracellular concentration of hydrogen peroxide and catalase during aerobic growth of $\Delta oxyR$ *E. coli*. Experimental conditions were as described in the legend to Fig. 1.

TABLE 2. Oxidative damage in aerobically growing *E. coli*^a

Phase and strain	H ₂ O ₂ concn (μ M)	Mutation frequency ^b	Protein carbonyl concn (nmol/mg of protein)	TBARS concn (nmol/mg of protein)
Lag				
AB1157 (WT)	0.13 \pm 0.01	5 \pm 3	0.6 \pm 0.1	0.13 \pm 0.05
BGF611 (<i>katG</i>)	0.21 \pm 0.01*	3 \pm 3	0.5 \pm 0.1	0.26 \pm 0.04*
RK4936 (WT)	0.12 \pm 0.01	3 \pm 2	0.6 \pm 0.1	0.15 \pm 0.06
TA4112 ($\Delta oxyR$)	0.5 \pm 0.02**	50 \pm 20**	0.50 \pm 0.03	0.33 \pm 0.06**
TA4482 (<i>ahpD5</i>)	0.13 \pm 0.04	60 \pm 3**	0.6 \pm 0.1	0.40 \pm 0.04**
Exponential				
AB1157 (WT)	0.25 \pm 0.03	17 \pm 1	1.5 \pm 0.3	0.17 \pm 0.04
BGF611 (<i>katG</i>)	0.31 \pm 0.03	16 \pm 1	1.6 \pm 0.2	0.10 \pm 0.01
RK4936 (WT)	0.20 \pm 0.05	13 \pm 1	1.6 \pm 0.2	0.18 \pm 0.02
TA4112 ($\Delta oxyR$)	0.95 \pm 0.05**	130 \pm 10**	1.7 \pm 0.2	0.30 \pm 0.08**
TA4482 (<i>ahpD5</i>)	0.25 \pm 0.05	60 \pm 2**	1.5 \pm 0.3	0.16 \pm 0.02
Stationary				
AB1157 (WT)	0.18 \pm 0.02	5 \pm 1	1.1 \pm 0.1	0.20 \pm 0.04
BGF611 (<i>katG</i>)	0.17 \pm 0.02	4 \pm 2	0.9 \pm 0.1	0.20 \pm 0.08
RK4936 (WT)	0.15 \pm 0.02	7 \pm 1	1.0 \pm 0.1	0.10 \pm 0.02
TA4112 ($\Delta oxyR$)	0.50 \pm 0.02**	40 \pm 6**	1.0 \pm 0.1	0.14 \pm 0.04
TA4482 (<i>ahpD5</i>)	0.20 \pm 0.01	20 \pm 2**	1.0 \pm 0.1	0.17 \pm 0.01

^a * and **, $P < 0.01$ and $P < 0.001$, respectively, compared to AB1157 under the same growth conditions.

^b Number of Rif^r bacteria per 10⁹ cells.

^c WT, wild type.

estingly, *rpoS* deficiency did not further affect the catalase or H₂O₂ concentration in either the wild-type or the $\Delta oxyR$ strain at all stages of growth (14).

The higher values for the H₂O₂ concentration in the $\Delta oxyR$ strain TA4112 and the smaller-than-expected effect of *katG* on H₂O₂ levels (Fig. 1B) imply that other *oxyR*-regulated activities could act in concert with HP-I to limit intracellular H₂O₂. One candidate for such an activity is the AhpFC alkyl hydroperoxide reductase (20), which was recently reported to use H₂O₂ as a substrate in vitro (31). However, the *ahpD5* strain TA4482 had levels of intracellular H₂O₂ concentration and catalase activity that were the same as those measured in wild-type *E. coli* (data not shown) (cf. Fig. 1A).

Oxidative damage to cellular components by sublethal increases in H₂O₂. The increased concentration of H₂O₂ in exponentially growing AB1157 and in the *katG* mutant and $\Delta oxyR$ strain prompted us to evaluate the occurrence of oxidative damage in those cells. Oxidative protein damage was monitored by assaying for protein carbonyls (23), membrane damage was estimated as TBARS concentration, and spontaneous mutations were scored as an indirect indicator of DNA damage. The growth rates of wild-type, *katG* mutant, *ahpD5*, and $\Delta oxyR$ strains were determined for lag, exponential, and stationary phases.

The frequencies of spontaneous mutations in the wild-type, *katG* mutant, and $\Delta oxyR$ strains were two- to fivefold higher during exponential growth than those during lag or stationary phase (Table 2). The mutation frequency in the $\Delta oxyR$ strain TA4112 was ~ 10 times higher than that of the wild type at each stage of growth (Table 2). Unexpectedly, the *katG* mutant displayed wild-type mutation rates at all stages of growth (Table 2). Although alkyl hydroperoxide reductase activity did not detectably affect H₂O₂ levels (see above), the spontaneous mutation frequency for the *ahpD5* strain TA4482 was elevated at each stage of growth and was as high in lag phase as in

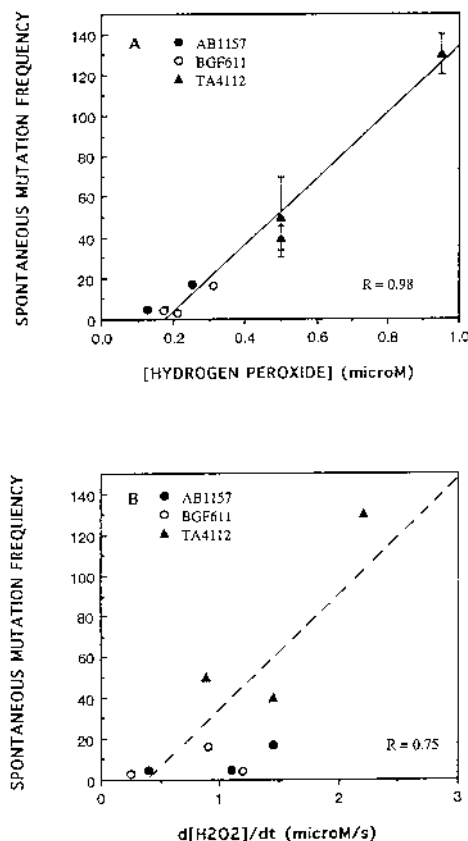


FIG. 6. Spontaneous mutation frequency as a function of the intracellular concentration of H₂O₂ (A) or the rate of H₂O₂ production (B) in wild-type *E. coli*, *katG* strain BGF611, and $\Delta oxyR$ strain TA4112. Error bars show SEMs.

exponential phase (Table 2). For all the strains except TA4482, the mutation frequency directly correlated with the intracellular steady-state concentration of H₂O₂ (Fig. 6A) rather than with its rate of production (Fig. 6B), although there was still an association ($R = 0.75$) in the latter comparison. As found by others (35), the introduction into TA4112 of multicopy plasmids bearing either *katG* or *oxyR* decreased the frequency of spontaneous mutations to wild-type values (14).

As a further check of the contribution of H₂O₂ to the increased mutation rate in the $\Delta oxyR$ strain, AB1157 and TA4112 were grown to exponential phase (20 h) and tested for the occurrence of Rif^r colonies under low-oxygen conditions. Under these circumstances, low mutation frequencies of 0 and 2 ± 1 colonies/10⁹ cells were obtained for the wild-type and $\Delta oxyR$ strains, respectively. Both values were significantly lower than the value for the exponentially growing wild-type strain under aerobic conditions (Table 2). A mutator effect of $\Delta oxyR$ mutations and its aerobic dependence were previously reported (15, 34), but this effect was not assessed as a function of the cell growth state.

Although protein oxidative damage (estimated as protein carbonyls) varied with the growth phase, there was never a significant difference in the carbonyl content between the wild-type and any of the mutant strains (Table 2). For lipid damage estimated as TBARS concentration (Table 2), the *katG*, *ahp* $\Delta 5$, and $\Delta oxyR$ strains all exhibited higher levels than the wild-type strains during lag phase. During exponential phase, the $\Delta oxyR$ strain had higher TBARS levels than did the wild-type parent or *ahp* $\Delta 5$ strains, while during lag phase, only the

ahp $\Delta 5$ strain had TBARS levels significantly higher than those of its wild-type parent. Thus, even when alkyl hydroperoxide reductase is functional, excessive accumulation of H₂O₂ evidently leads to the accumulation of lipid peroxides, as can normal H₂O₂ levels in the absence of *ahpFC*.

The growth rates of exponential-phase cultures also reflected the intracellular concentration of H₂O₂. Doubling times for strains AB1157 and BGF611 were 45 ± 5 and 45 ± 7 min, respectively. For strains RK4936, TA4482 (*ahp* $\Delta 5$), and TA4112 ($\Delta oxyR$), the doubling times were 45 ± 4 , 45 ± 3 , and 70 ± 5 min, respectively, which suggests a modest cytostatic effect of the approximately fourfold-increased steady-state concentration of H₂O₂ in strain TA4112.

DISCUSSION

We have shown here (i) that *E. coli* maintains the intracellular concentration of H₂O₂ within a surprisingly narrow range (~ 0.2 μ M) although the metabolic generation of H₂O₂ varies >10 -fold during growth; (ii) that a significant amount of this homeostatic control during exponential growth seems to be accomplished by the transcriptional regulation of the *katG*-encoded HP-I; (iii) that the variation of HP-I catalase levels reflects a continuous variation in the degree of OxyR activation; (iv) that the protective role of OxyR activation is a population-wide phenomenon, rather than one restricted to occasional cells undergoing more intense oxidative stress; (v) that the physiological trigger point for OxyR seems to lie at ~ 0.2 μ M, the level above which *katG* up-regulation is observed; and (vi) that *ahpFC* plays an antimutator role but does not regulate H₂O₂ levels. These key points are discussed below.

It is noteworthy that the $\Delta oxyR$ mutation led to higher levels of H₂O₂ in all phases of cell growth than did inactivation of the *oxyR*-regulated *katG* gene (Fig. 1B and 2 and Table 2). Since the *ahp* $\Delta 5$ mutation did not significantly affect H₂O₂ levels, still other *oxyR*-regulated functions must play a role in limiting the concentration of this metabolite.

The increased concentration of H₂O₂ upon entering exponential growth could be associated with the occurrence of oxidative DNA damage, as evidenced by the increased frequency of spontaneous mutations, and was associated with growth impairment in TA4112. Lipid peroxidation damages (estimated here as TBARS concentration) could exert a mutagenic effect by leading to DNA modification (4). Such an effect would be consistent with the elevated TBARS levels and mutation frequencies for both the $\Delta oxyR$ and *ahp* $\Delta 5$ strains in lag phase and for the $\Delta oxyR$ strain in exponential growth (Table 2). The continued mutator effect of *ahp* $\Delta 5$ in exponential and stationary phases indicates that alkyl hydroperoxide reductase eliminates other mutagenic metabolites not detectable by the TBARS assay.

The present observations suggest a set point near 0.2 μ M for triggering OxyR activation. Surprisingly, the rate of H₂O₂ production correlated more closely with OxyR activity than did the intracellular H₂O₂ concentration. OxyR in vivo may be sensitive to changes in the H₂O₂ levels rather than to the absolute values, analogous to signal processing in chemotaxis (22).

The increased occurrence of oxidative damage in cells lacking the *oxyR*-dependent response to H₂O₂ and in wild-type bacteria during exponential growth suggested that the 0.2 μ M value for the intracellular concentration of H₂O₂ is about optimal in balancing cell growth with defense needs. Interestingly, twofold increases in the intracellular H₂O₂ concentration accompanying inactivation of antioxidant enzymes caused irreversible damage in rat tissues (10, 11), indicative of the

potential for biologically important effects due to relatively small changes in the cellular oxidant status.

The complete loss of *katG* transcriptional regulation in the $\Delta oxyR rpoS$ double mutant was unexpected, in part because of the near-normal pattern seen for the *rpoS* single mutant (Fig. 2). It seems possible that *oxyR*⁺ might somehow compensate for the missing regulation in the *rpoS* strain, especially in stationary phase. The activation of OxyR to compensate for an *rpoS* mutation would imply that significant amounts of H₂O₂ are generated in stationary phase in *rpoS* cells, perhaps because HP-II activity remains below normal levels. More σ^{70} -containing RNA polymerase could also remain in the *rpoS* mutants than in wild-type cells to support OxyR-dependent transcription (19). Conversely, the higher level of expression of *katG::lacZ* at all times in the *oxyR* mutant strain compared to the *oxyR rpoS* double mutant indicates that *rpoS* can contribute to HP-I expression even during exponential growth.

On the basis of this paper and previous reports by Imlay and Fridovich (18) and by us (13), it is possible to draw an integrated scheme for the metabolism of oxygen free radicals in *E. coli*. Superoxide (O₂⁻) production as a by-product of respiratory activity would proceed at a rate of $\sim 4 \times 10^{-6}$ M s⁻¹ (18) in the surroundings of the internal surface of the cell membrane, where ubiquinone and NADH-dehydrogenase are located. O₂⁻ diffuses within the cytosolic compartment where it can either react with metal targets or be metabolized by superoxide dismutases to yield hydrogen peroxide (H₂O₂) at a rate of $\sim 10^{-6}$ M s⁻¹ (13). The intracellular concentration of H₂O₂ is determined by its rate of diffusion out of the cell, its destruction by intracellular catalase and other peroxidases (mainly HP-I), and its reaction with Fe²⁺ or other metals to produce HO[•]. Under most conditions during aerobic growth the intracellular concentrations of O₂⁻ and H₂O₂ are kept at $\sim 10^{-10}$ and $\sim 10^{-7}$ M, respectively (13, 18). This regulation is due to *oxyR*-dependent regulation of HP-I activity and perhaps to *soxRS*-dependent regulation of Mn-superoxide dismutase (12). The rate of oxidation of cellular components by O₂⁻, H₂O₂, or HO[•] will depend on the overall metabolic status of the cell and on the concentrations of reactive oxygen species and transition metals such as iron. Finally, the cellular content of oxidized lipids, proteins, and DNA in a given cell will also depend on the activity of the repair and degradation systems for damaged molecules. It also seems possible that changes in the cellular permeability to H₂O₂ and O₂⁻ might constitute important parameters, but such changes have not been documented.

ACKNOWLEDGMENTS

We are grateful to Elena Hidalgo for helpful discussions and to Roberto Kolter for his advice for testing *rpoS* phenotypes. We also thank Gisela Storz (National Institute of Child Health and Human Development) for providing us with strains TA4112 and TA4482. We appreciate the helpful comments of a referee.

This work was supported by grants to B.D. from the National Institutes of Health (CA37831) and the Amyotrophic Lateral Sclerosis Association. B.G.-F. acknowledges the generous support of a fellowship from the Pew Charitable Trusts.

REFERENCES

- Asakawa, T., and S. Matsushita. 1980. Coloring conditions of thiobarbituric acid test for detecting lipid hydroperoxides. *Lipids* 15:137-140.
- Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2:95-113.
- Chance, B., H. Sies, and A. Boveris. 1979. Hydroperoxides metabolism in mammalian organs. *Physiol. Rev.* 59:527-605.
- Chaudhary, A. K., M. Nokubo, G. R. Redy, S. N. Yeola, J. D. Morrow, I. A. Blair, and L. J. Marnett. 1994. Detection of endogenous malondialdehyde-deoxyguanosine adducts in human liver. *Science* 265:1580-1582.
- Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* 41:753-762.
- Claiborne, A., and I. Fridovich. 1979. Purification of the *o*-dianisidine peroxidase from *Escherichia coli* B. Physicochemical characterization and analysis of its dual catalytic and peroxidatic activities. *J. Biol. Chem.* 254:4245-4252.
- Claiborne, A., D. P. Malinowski, and I. Fridovich. 1979. Purification and characterization of hydroperoxidase II of *Escherichia coli* B. *J. Biol. Chem.* 254:11664-11668.
- Fields, R., and H. B. F. Dixon. 1971. Micro method for determination of reactive carbonyl groups in proteins and peptides, using 2,4-dinitrophenylhydrazine. *Biochem. J.* 121:587-593.
- Fraga, C. G., B. E. Leibovich, and A. L. Tappel. 1987. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes. *Free Radical Biol. Med.* 3:119-123.
- González-Flecha, B., J. C. Cutrin, and A. Boveris. 1993. Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to *in vivo* ischemia-reperfusion. *J. Clin. Invest.* 91:456-464.
- González-Flecha, B., P. Evelson, N. Sterin-Speziale, and A. Boveris. 1993. Hydrogen peroxide metabolism and oxidative stress in cortical, medullary and papillary zones of rat kidney. *Biochim. Biophys. Acta* 1157:155-161.
- González-Flecha, B., and B. Dimple. 1994. Intracellular generation of superoxide as a by-product of *Vibrio harveyi* luciferase expressed in *Escherichia coli*. *J. Bacteriol.* 176:2293-2299.
- González-Flecha, B., and B. Dimple. 1995. Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. *J. Biol. Chem.* 270:13681-13687.
- González-Flecha, B., and B. Dimple. Unpublished data.
- Greenberg, G., and B. Dimple. 1988. Overproduction of superoxide-scavenging enzymes in *Escherichia coli* suppresses spontaneous mutagenesis and sensitivity to redox-cycling agents in *oxyR*⁻ mutants. *EMBO J.* 7:2611-2617.
- Hassan, H. M., and I. Fridovich. 1978. Regulation of the synthesis of catalase and peroxidase in *Escherichia coli*. *J. Biol. Chem.* 253:6445-6450.
- Huisman, G., and R. Kolter. 1994. Regulation of gene expression at the onset of stationary phase in *Escherichia coli*, p. 21-40. In P. J. Piggot, C. P. Moran, Jr., and P. Youngman (ed.), *Regulation of bacterial differentiation*. American Society for Microbiology, Washington, D.C.
- Imlay, J. A., and I. Fridovich. 1991. Assay of metabolic superoxide production in *Escherichia coli*. *J. Biol. Chem.* 266:6957-6965.
- Ivanova, A., C. Miller, G. Glinsky, and A. Eisenstark. 1994. Role of *rpoS* (*katF*) in *oxyR*-independent regulation of hydroperoxidase I in *Escherichia coli*. *Mol. Microbiol.* 12:571-578.
- Jacobson, F. S., R. W. Morgan, M. F. Christman, and B. N. Ames. 1989. An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. Purification and properties. *J. Biol. Chem.* 264:1488-1496.
- Johnson, G. D. 1953. Correlation of color and constitution. I. 2,4-Dinitrophenylhydrazones. *J. Am. Chem. Soc.* 75:2720-2725.
- Koshland, D. E., Jr. 1981. Biochemistry of sensing and adaptation in a simple bacterial system. *Annu. Rev. Biochem.* 50:765-781.
- Levine, R. L., J. A. Williams, E. R. Stadtman, and E. R. Shacter. 1994. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* 233:346-357.
- Loewen, P. C. 1984. Isolation of catalase-deficient *Escherichia coli* mutants and genetic mapping of *kate*, a locus that affects catalase activity. *J. Bacteriol.* 157:622-626.
- Loewen, P. C., and B. L. Triggs. 1984. Genetic mapping of *kate*, a locus that with *kate* affects the synthesis of a second catalase species in *Escherichia coli*. *J. Bacteriol.* 160:668-675.
- Loewen, P. C., J. Switala, and B. L. Triggs-Raine. 1985. Catalases HPI and HPII in *Escherichia coli* are induced independently. *Arch. Biochem. Biophys.* 243:144-149.
- Lopez-Barea, J., and C. Y. Lee. 1979. Mouse-liver glutathione reductase. Purification, kinetics, and regulation. *Eur. J. Biochem.* 98:487-489.
- Lowry, O. H., A. L. Rosebrough, A. L. Farr, and R. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Miller, J. H. 1992. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mulvey, M. L., P. A. Sorby, B. L. Triggs-Raine, and P. C. Loewen. 1988. Cloning and physical characterization of *kate* and *kateF* required for catalase HPII expression in *Escherichia coli*. *Gene* 73:337-345.
- Nimura, Y., L. B. Poole, and V. Massey. 1995. *Amphibacillus xylanus* NADH oxidase and *Salmonella typhimurium* alkyl-hydroperoxide reductase flavoprotein components show extremely high scavenging activity for both alkyl hydroperoxide and hydrogen peroxide in the presence of *S. typhimurium* alkyl-hydroperoxide reductase 22-kDa protein component. *J. Biol. Chem.* 270:25645-25650.
- Pearson, M. L. 1972. The role of adenosine 3',5'-cyclic monophosphate in the growth of bacteriophage lambda. *Virology* 49:605-609.

33. Sak, B. D., A. Eisenstark, and D. Touati. 1989. Exonuclease III and the catalase-hydroperoxidase II in *Escherichia coli* are both regulated by the *katF* gene product. *Proc. Natl. Acad. Sci. USA* **86**:3271–3275.
34. Storz, G., M. F. Christman, H. Sies, and B. Ames. 1987. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **84**:8917–8921.
35. Storz, G., L. A. Tartaglia, and B. N. Ames. 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* **248**: 189–194.
36. Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa. 1989. Molecular cloning and nucleotide sequencing of *oxyR*, the positive regulatory gene of a regulon for an adaptive response to oxidative stress in *Escherichia coli*: homologies between OxyR protein and a family of bacterial activator proteins. *Mol. Gen. Genet.* **218**:371–376.
37. Tartaglia, L. A., G. Storz, and B. N. Ames. 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. *J. Mol. Biol.* **210**:709–719.
38. Triggs-Raine, B. L., and P. C. Loewen. 1987. Physical characterization of *katG*, encoding catalase HPI of *Escherichia coli*. *Gene* **52**:121–128.
39. Twigg, A. J., and D. Sherratt. 1980. Trans-complementable copy-number mutants of plasmid ColE1. *Nature* **283**:216–218.
40. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.