JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 382–388
0021-9193/97/$04.00+0

Vol. 179, No. 2

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

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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 H_2O_2 generation. We addressed this issue by studying the regulation of the intracellular concentration of H_2O_2 in aerobically growing *Escherichia coli*. Intracellular H_2O_2 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 H_2O_2 generation. The OxyR-regulated alkyl hydroperoxide reductase encoded by *ahpFC* did not detectably affect H_2O_2 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 *ahp*Δ*5* 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 H_2O_2 produced by normal aerobic metabolism. The activation of the *oxyR* regulon in this process occurs at much lower levels of H_2O_2 (~10^{-7} M) than those reported for *oxyR* activation by exogenous H_2O_2 (~10^{-5} M).

The intracellular concentration of hydrogen peroxide (H_2O_2) depends on the rates of H_2O_2 production and consumption. Physiological production of superoxide anion (O_2^-) and the resulting H_2O_2 in *Escherichia coli* is mainly related to respiratory activity (13, 18) and varies considerably along the bacterial growth curve (13). Detoxification of H_2O_2 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 H_2O_2 (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 H_2O_2-inducible proteins in *Salmonella typhimurium* and *E. coli* (5, 15). *S. typhimurium* and *E. coli* Δ*oxyR* mutants are hypersensitive to H_2O_2 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 H_2O_2 is responsible for the elevated mutation rate. However, the molecular mechanisms that limit H_2O_2 toxicity in normal cells are unclear. Specifically, the possibility that the Δ*oxyR* mutator effect applies only to rare individual cells with dramatically increased H_2O_2 levels rather than to the population as a whole remains. For example, elevated H_2O_2 levels in individual cells could arise from damage to components of the respiratory chain (13).

To define the basic features that affect H_2O_2 levels in *E. coli*, we have previously characterized the physiological sources of H_2O_2 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*::Tn*10* allele from strain UM257 into strain BGF933. Strains BGF611 and BGF711 were constructed by transduction of *katG17*::Tn*10* and *katE12*::Tn*10* from UM202 and UM254, respectively, into AB1157. The genotype of *rpoS* strains was confirmed by the H_2O_2-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

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic	Source or reference
Strains		
AB1157	F$^-$ *thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx-33*	Laboratory stock
BGF611	As AB1157, but *katG17*::Tn*10*	This work
BGF711	As AB1157, but *katE12*::Tn*10*	This work
BGF931	As RK4936, but λ[Φ(*katG′*::*lacZ*)]	13
BGF933	As TA4112, but λ[Φ(*katG′*::*lacZ*)	13
BGF1033	As BGF933, but *rpoS13*::Tn*10*	This work
BGF1041	As AB1157, but *rpoS13*::Tn*10* λ[Φ(*katG′*::*lacZ*)]	This work
MC4100	Δ(*lac*)*U169 rpsL*	Laboratory stock
MP180	HfrH *thi*	32
RK4936	*araD139* (*argF-lac*)*205 glbB5301 non-* (*gyrA219*/*relA1 rpsL150 metE70 btuB*::Tn*10*)	Laboratory stock
RR1	*pro leu rpsL hsdM hsdR endI lacY*	2
TA4112	As RK4936, but Δ(*oxyR btuB*)*3*	5
TA4482	As RK4936, but *ahp*Δ*5 zij-602*::Tn*10*	37
UM202	As MP180, but *katG17*::Tn*10*	26
UM257	As RR1, but *rpoS13*::Tn*10 katG15 thyA*	30
UM254	As RR1, but *katE12*::Tn*10 katG15 thyA*	30
Plasmids		
pAT153	Vector Ap^r Tc^r	39
pUC18	Vector Ap^r	40
pBT22	pAT153 containing *katG*	38
pKT181	pUC18 containing *oxyR*	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 H_2O_2 were measured by the procedures previously described (12). Briefly, bacterial cultures taken at the indicated times were pelleted and resuspended at 10^6 cells/ml in phosphate-buffered saline. H_2O_2 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*), H_2O_2 concentration in the supernatant was measured by a horseradish peroxidase-scopoletin assay (13). Complete equilibration of the intra- and extracellular H_2O_2 level occurs within 10 min in this assay (13).

The rate of H_2O_2 production was calculated from the experimental values of intracellular H_2O_2 and catalase concentration by using the steady-state assumption that the rate of H_2O_2 production equals the rate of H_2O_2 elimination. The rate of H_2O_2 consumption could be calculated because catalase activity is the major activity eliminating H_2O_2 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 H_2O_2 by catalase (k_1 for bacterial catalase = 0.5×10^6 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 H_2O_2 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 H_2O_2 per min. β-Galactosidase activity in sodium dodecyl sulfate-CH_3Cl-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 $\varepsilon_{390\text{-}350}$ 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 H_2O_2 in *E. coli*. Our previous studies (13) showed that the rate of H_2O_2 production increases >10-fold during exponential growth. We therefore measured the concentration of H_2O_2 in intact *E. coli* AB1157 as a function of aerobic growth in rich medium. These measurements revealed that the intracellular H_2O_2 concentration ranged from 0.13 to 0.25 μM (Fig. 1A), which points to the existence of regulatory systems that maintain intracellular levels of H_2O_2 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 H_2O_2 concentration (Fig. 1A). The H_2O_2 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 H_2O_2 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 H_2O_2 levels, we measured this metabolite in the mutant strains. The intracellular concentration of H_2O_2 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 H_2O_2 levels during exponential growth. However, the increase in the H_2O_2 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

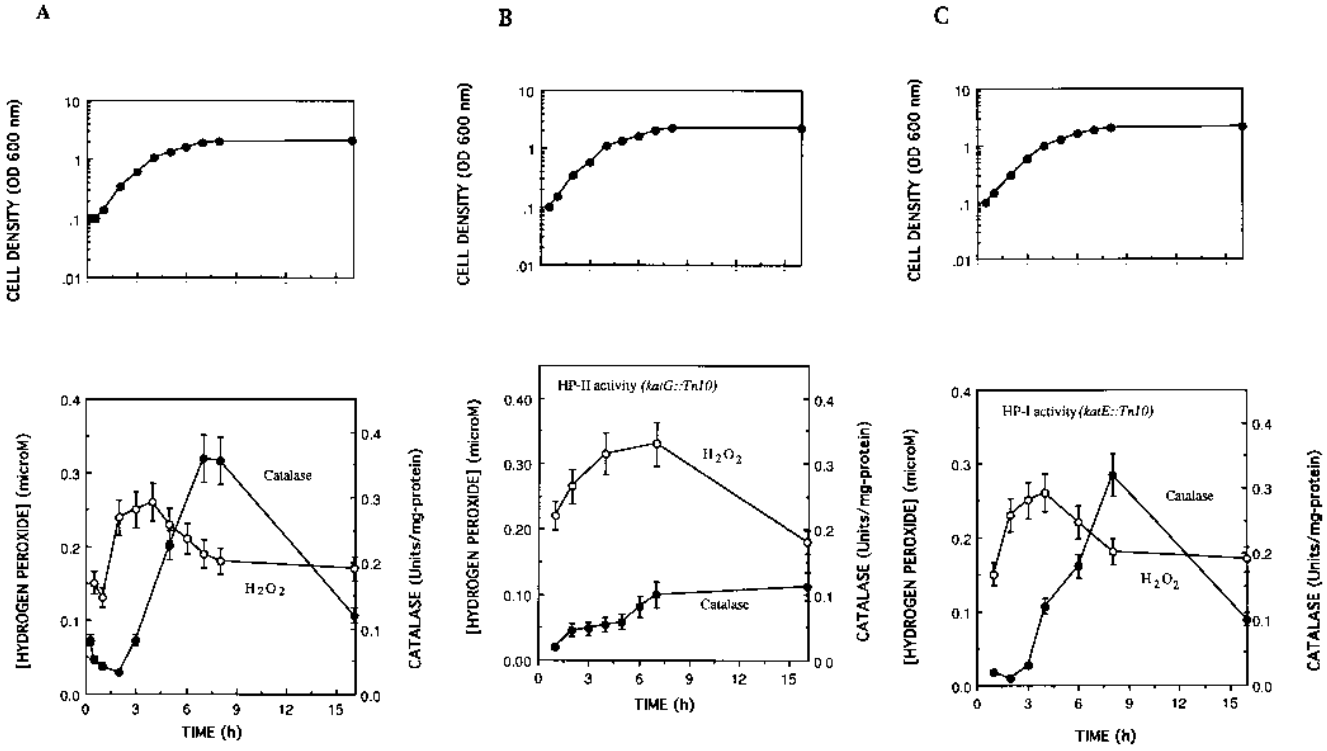

FIG. 1. Intracellular concentration of hydrogen peroxide and catalase during aerobic growth. Overnight cultures of *E. coli* AB1157 (wild type) (A), BGF611 (*katG* mutant) (B), or BGF711 (*katE* mutant) (C) were diluted 1/100 in fresh LB and incubated at 37°C with gentle shaking (100 rpm). At the indicated times, samples were taken to assay catalase activity (filled circles) and H_2O_2 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, glutathione 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 H_2O_2 production or to the steady-state level of H_2O_2. We therefore attempted to correlate these parameters with *katG* expression or with catalase activity. The rate of H_2O_2 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 H_2O_2 did not correlate well with either *katG′*::*lacZ* expression (14) or catalase activity (Fig. 4B). We did not attempt to correlate H_2O_2 production and catalase

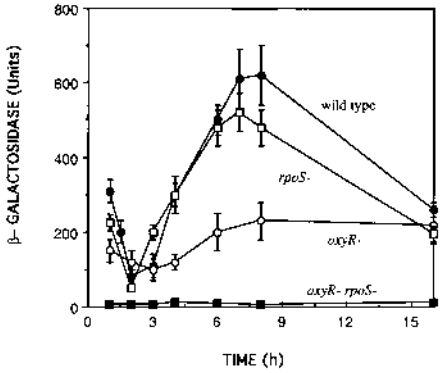

FIG. 2. Induction of a *katG′*::*lacZ* operon fusion in aerobically growing *E. coli*. Cultures of strains BGF931 (wild type [filled circles]), BGF933 (Δ*oxyR* [open circles]), BGF1033 (Δ*oxyR rpoS* mutant [filled squares]), and BGF1041 (*rpoS* mutant [open squares]) were grown at 37°C in LB. Samples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

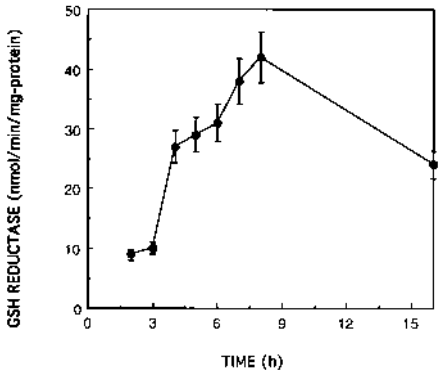

FIG. 3. Glutathione (GSH) reductase activity during aerobic growth of *E. coli* AB1157. Error bars show SEMs.

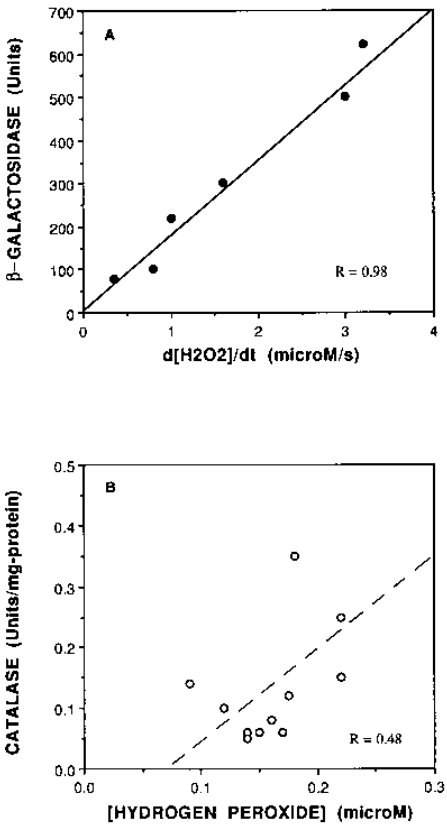

FIG. 4. β-Galactosidase activity from a *katG′*::*lacZ* operon fusion as a function of the rate of H_2O_2 generation (A) and catalase activity as a function of the intracellular concentration of H_2O_2 (B) during 2 to 16 h of growth.

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 Δ*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 H_2O_2 concentrations in the Δ*oxyR* strain were increased to values ranging from 0.5 to 1 μM (Fig. 5), much higher than those found for the *katG* mutant (Fig. 1B). Interestingly, *rpoS* deficiency did not further affect the catalase or H_2O_2 concentration in either the wild-type or the Δ*oxyR* strain at all stages of growth (14).

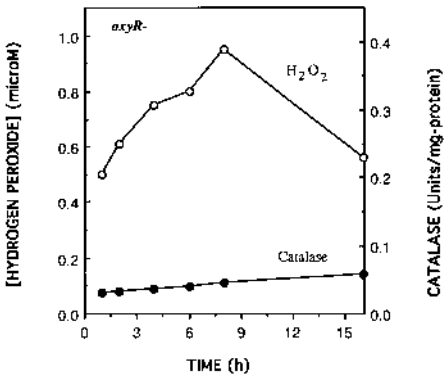

FIG. 5. Intracellular concentration of hydrogen peroxide and catalase during aerobic growth of Δ*oxyR E. coli*. Experimental conditions were as described in the legend to Fig. 1.

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

Phase and strain	H_2O_2 concn (μM)	Mutation frequency[b]	Protein carbonyl concn (nmol/mg of protein)	TBARS concn (nmol/mg of protein)
Lag				
AB1157 (WT[c])	0.13 ± 0.01	5 ± 3	0.6 ± 0.1	0.13 ± 0.05
BGF611 (*katG*)	0.21 ± 0.01*	3 ± 3	0.5 ± 0.1	0.26 ± 0.04*
RK4936 (WT)	0.12 ± 0.01	3 ± 2	0.6 ± 0.1	0.15 ± 0.06
TA4112 (Δ*oxyR*)	0.5 ± 0.02**	50 ± 20**	0.50 ± 0.03	0.33 ± 0.06**
TA4482 (*ahp*Δ*5*)	0.13 ± 0.04	60 ± 3**	0.6 ± 0.1	0.40 ± 0.04**
Exponential				
AB1157 (WT)	0.25 ± 0.03	17 ± 1	1.5 ± 0.3	0.17 ± 0.04
BGF611 (*katG*)	0.31 ± 0.03	16 ± 1	1.6 ± 0.2	0.10 ± 0.01
RK4936 (WT)	0.20 ± 0.05	13 ± 1	1.6 ± 0.2	0.18 ± 0.02
TA4112 (Δ*oxyR*)	0.95 ± 0.05**	130 ± 10**	1.7 ± 0.2	0.30 ± 0.08**
TA4482 (*ahp*Δ*5*)	0.25 ± 0.05	60 ± 2**	1.5 ± 0.3	0.16 ± 0.02
Stationary				
AB1157 (WT)	0.18 ± 0.02	5 ± 1	1.1 ± 0.1	0.20 ± 0.04
BGF611 (*katG*)	0.17 ± 0.02	4 ± 2	0.9 ± 0.1	0.20 ± 0.08
RK4936 (WT)	0.15 ± 0.02	7 ± 1	1.0 ± 0.1	0.10 ± 0.02
TA4112 (Δ*oxyR*)	0.50 ± 0.02**	40 ± 6**	1.0 ± 0.1	0.14 ± 0.04
TA4482 (*ahp*Δ*5*)	0.20 ± 0.01	20 ± 2**	1.0 ± 0.1	0.17 ± 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 10^9 cells.
[c] WT, wild type.

The higher values for the H_2O_2 concentration in the Δ*oxyR* strain TA4112 and the smaller-than-expected effect of *katG* on H_2O_2 levels (Fig. 1B) imply that other *oxyR*-regulated activities could act in concert with HP-I to limit intracellular H_2O_2. One candidate for such an activity is the AhpFC alkyl hydroperoxide reductase (20), which was recently reported to use H_2O_2 as a substrate in vitro (31). However, the *ahp*Δ*5* strain TA4482 had levels of intracellular H_2O_2 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 H_2O_2. The increased concentration of H_2O_2 in exponentially growing AB1157 and in the *katG* mutant and Δ*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, *ahp*Δ*5*, and Δ*oxyR* strains were determined for lag, exponential, and stationary phases.

The frequencies of spontaneous mutations in the wild-type, *katG* mutant, and Δ*oxyR* strains were two- to fivefold higher during exponential growth than those during lag or stationary phase (Table 2). The mutation frequency in the Δ*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 H_2O_2 levels (see above), the spontaneous mutation frequency for the *ahp*Δ*5* strain TA4482 was elevated at each stage of growth and was as high in lag phase as in

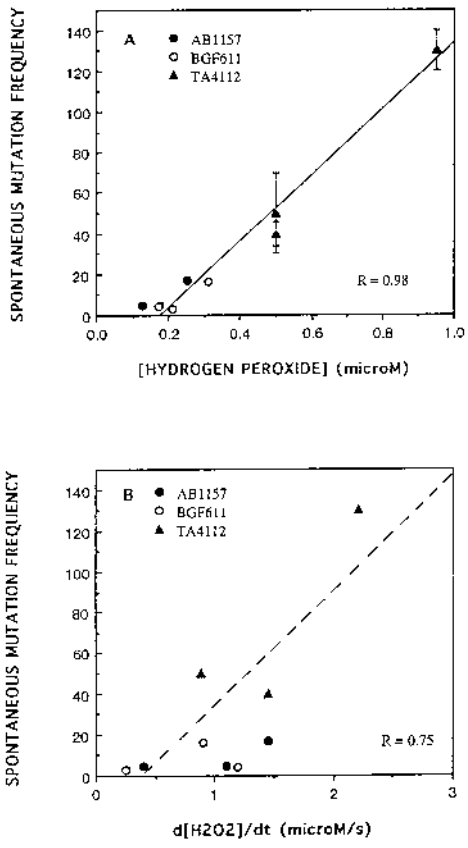

FIG. 6. Spontaneous mutation frequency as a function of the intracellular concentration of H_2O_2 (A) or the rate of H_2O_2 production (B) in wild-type *E. coli, katG* strain BGF611, and Δ*oxyR* strain TA4112. Error bars show SEMs.

exponential phase (Table 2). For all the strains except TA4482, the mutation frequency directly correlated with the intracellular steady-state concentration of H_2O_2 (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 H_2O_2 to the increased mutation rate in the Δ*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/10^9 cells were obtained for the wild-type and Δ*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 Δ*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*, *ahpΔ5*, and Δ*oxyR* strains all exhibited higher levels than the wild-type strains during lag phase. During exponential phase, the Δ*oxyR* strain had higher TBARS levels than did the wild-type parent or *ahpΔ5* strains, while during lag phase, only the *ahpΔ5* strain had TBARS levels significantly higher than those of its wild-type parent. Thus, even when alkyl hydroperoxide reductase is functional, excessive accumulation of H_2O_2 evidently leads to the accumulation of lipid peroxides, as can normal H_2O_2 levels in the absence of *ahpFC*.

The growth rates of exponential-phase cultures also reflected the intracellular concentration of H_2O_2. Doubling times for strains AB1157 and BGF611 were 45 ± 5 and 45 ± 7 min, respectively. For strains RK4936, TA4482 (*ahpΔ5*), and TA4112 (Δ*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 H_2O_2 in strain TA4112.

DISCUSSION

We have shown here (i) that *E. coli* maintains the intracellular concentration of H_2O_2 within a surprisingly narrow range (~0.2 μM) although the metabolic generation of H_2O_2 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 μM, the level above which *katG* up-regulation is observed; and (vi) that *ahpFC* plays an antimutator role but does not regulate H_2O_2 levels. These key points are discussed below.

It is noteworthy that the Δ*oxyR* mutation led to higher levels of H_2O_2 in all phases of cell growth than did inactivation of the *oxyR*-regulated *katG* gene (Fig. 1B and 2 and Table 2). Since the *ahpΔ5* mutation did not significantly affect H_2O_2 levels, still other *oxyR*-regulated functions must play a role in limiting the concentration of this metabolite.

The increased concentration of H_2O_2 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 Δ*oxyR* and *ahpΔ5* strains in lag phase and for the Δ*oxyR* strain in exponential growth (Table 2). The continued mutator effect of *ahpΔ5* 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 μM for triggering OxyR activation. Surprisingly, the rate of H_2O_2 production correlated more closely with OxyR activity than did the intracellular H_2O_2 concentration. OxyR in vivo may be sensitive to changes in the H_2O_2 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 H_2O_2 and in wild-type bacteria during exponential growth suggested that the 0.2 μM value for the intracellular concentration of H_2O_2 is about optimal in balancing cell growth with defense needs. Interestingly, twofold increases in the intracellular H_2O_2 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 Δ*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 H_2O_2 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 (O_2^-) 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. O_2^- diffuses within the cytosolic compartment where it can either react with metal targets or be metabolized by superoxide dismutases to yield hydrogen peroxide (H_2O_2) at a rate of $\sim 10^{-6}$ M s^{-1} (13). The intracellular concentration of H_2O_2 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 Fe^{2+} or other metals to produce $HO^{\cdot}$. Under most conditions during aerobic growth the intracellular concentrations of O_2^- and H_2O_2 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 O_2^-, H_2O_2, or $HO^{\cdot}$ 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 H_2O_2 and O_2^- 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 Amyotropic Lateral Sclerosis Association. B.G.-F. acknowledges the generous support of a fellowship from the Pew Charitable Trusts.

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