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Metabolic Sources of Hydrogen Peroxide in Aerobically Growing *Escherichia coli**

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Exposure of cells to hydrogen peroxide (H_2O_2) mediates adaptive responses or oxidative damage, depending on the magnitude of the challenge. Determining the threshold for peroxide-mediated oxidative stress thus requires quantitation of the changes in endogenous H_2O_2 production. The intracellular steady-state concentrations of H_2O_2 were measured in intact *Escherichia coli* under different conditions. Compounds that block electron transport at NADH dehydrogenase (rotenone) or between ubiquinone and cytochrome *b* (antimycin) showed that univalent reduction of O_2 can occur at these sites *in vivo* to form superoxide anion (O_2^-), in agreement with reports for mammalian mitochondria. Mutational inactivation of different components of the respiratory chain showed that H_2O_2 production also depended on the energy status of the cell and on the arrangement of respiratory chain components corresponding to particular growth conditions. Production rates for O_2^- and H_2O_2 were linearly related to the number of active respiratory chains that reached maximal values during exponential growth. In the strains defective in respiratory chain components, catalase activity was regulated to compensate for changes in the H_2O_2 production rates, which maintained intracellular H_2O_2 at 0.1–0.2 μM during aerobic growth over a wide range of cell densities. The expression of a *katG′::lacZ* fusion (reporting transcriptional control of the catalase-hydroperoxidase I gene) was increased by H_2O_2 given either as a pulse or as a steady production. This response not only depended on the type and severity of the stimulus but was also strongly influenced by the growth phase of the cells.

Reactive by-products of oxygen, superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^\cdot$), are derived from sequential univalent reductions of molecular oxygen. These agents are produced continuously in aerobically growing cells (Chance *et al.*, 1979). In eukaryotic cells, the respiratory chain and cytochrome P-450 seem to be the significant intracellular sources of O_2^- (Chance *et al.*, 1979). H_2O_2 is produced by the superoxide dismutase-catalyzed dismutation of O_2^- in mitochondria and in the cytosol and by flavin oxidases in peroxisomes (Chance *et al.*, 1979). Intracellular O_2^- and H_2O_2 are kept at acceptably low concentrations by the action of antioxidant enzymes such as superoxide dismutase, catalase, and other peroxidases (Chance *et al.*, 1979; Sies, 1991).

"Oxidative stress" refers to imbalances between the production and disposal of oxygen radicals (Gerschman *et al.*, 1954; Sies, 1991). Oxidative stress has been associated with aging (Harman, 1991), carcinogenesis (Cerutti, 1985), and diverse clinical situations such as Alzheimer's disease (Luft, 1994; Yan, 1994) and cell damage due to ischemia-reperfusion (González-Flecha *et al.*, 1993). Oxidative stress is also exploited as a cytotoxic weapon during phagocytosis (Babior, 1991). H_2O_2 can be excreted by the acatalasic bacterium *Streptococcus sanguis* in amounts sufficient to prevent the growth of other organisms (Holmberg and Hallander, 1973). In plants, hydrogen peroxide from an oxidative burst in pathogen-infected cells may act as a signal for the induction of resistance in adjacent cells (Levine *et al.*, 1994). Environmental agents such as ionizing or near-UV radiations or numerous compounds that generate intracellular O_2^- (*e.g.* paraquat, plumbagin, and menadione) can cause oxidative stress (Sies, 1991; Kappus and Sies, 1981).

Genetic responses to oxidative stress occur in bacteria (Demple, 1991), yeast (Jamieson, 1994), and mammalian cell lines (Amstad *et al.*, 1994; Keyse and Tyrrel, 1989; Schulze-Osthoff and Baeuerle, 1994). *Escherichia coli* cells possess a specific defense against peroxides mediated by the transcriptional activator OxyR and another against superoxide, controlled by the two-stage *soxRS* system (Hidalgo and Demple, 1995). The OxyR regulon includes catalase-hydroperoxidase I, encoded by *katG*, a NADPH-dependent alkyl hydroperoxidase, encoded by *ahpFC*, glutathione reductase, encoded by *gorA*, a protective DNA binding protein, encoded by *dps*, and several other genes (Hidalgo and Demple, 1995). The expression of these genes is elevated in *E. coli* exposed to 5–100 μM H_2O_2 (Demple and Halbrook, 1983; Storz *et al.*, 1990; Demple, 1991; Hidalgo and Demple, 1995).

Despite intensive study of adaptive responses of bacteria to oxidative stress as cited above, no systematic analysis of the effects of growth state or the physiological threshold of oxidative stress required to trigger these responses has been reported. Imlay and Fridovich (1991a) estimated a steady-state O_2^- concentration of $\sim 10^{-10}$ M in *E. coli* by measuring the rate of superoxide production in isolated membranes. We present here a study of the physiological production and disposal of H_2O_2 in intact *E. coli*, measured by the rapid equilibration of intracellular H_2O_2 (which passes freely through membranes) (Chance *et al.*, 1979) with the surrounding medium. We have also analyzed intracellular sources of oxygen radicals, the effect of different types of oxidative stress on the steady-state H_2O_2 concentration, and the growth-dependent variation in the extent of *oxyR*-regulated H_2O_2 response.

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TABLE I
Bacterial strains used in this study

Strain	Genotype	Source or reference
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
BGF931	As RK4936, but λ[Φ(*katG'::lacZ*)]	This work
BGF933	As TA4112, but λ[Φ(*katG'::lacZ*)]	This work
GO103	As GR70N, but *zbg-2200*::kan Δ(*cydAB'*)*455*	Oden *et al.* (1990)
GO104	As GR70N, but Δ(*cyoABCDE*)*456*::kan	Calhoun *et al.* (1993)
GR70N	F⁻ *thi rpsL gal*	Green *et al.* (1988)
MC4100	Δ *(lac-pro) U169 rpsL*	Laboratory stock
MWC190	As GR70N, but *zej*-223::Tn*10 nuo* Man⁺	Calhoun and Gennis (1993)
MWC215	As GR70N, but *ndh*::Cm^r Man⁺	Calhoun and Gennis (1993)
MWC232	As GR70N, but *ndh*::Cm^r *zej*-223::Tn*10 nuo* Man⁻	Calhoun and Gennis (1993)
RK4936	*araD139 (argF-lac) 205 glbB5301 non-9 gyrA219/relA1 rpsL150 metE70 btuB::Tn10*	Laboratory stock
TA4112	As RK4936, but Δ(*oxyR-btuB*)*3*	Christman *et al.* (1985)

MATERIALS AND METHODS

Reagents—Antimycin A, ampicillin, rotenone, D-glucose, bovine cytochrome *c* type III, tetracycline, NADH, succinate, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)[1], scopoletin, bovine serum albumin, horseradish peroxidase type VI, glucose oxidase, bovine superoxide dismutase, and bovine catalase were purchased from Sigma.

Bacterial Strains and Growth Conditions—Table I lists the strains used in these studies. The bacteriophage λRS45[*bla'-'lacZ lacYA*⁺] (Simons *et al.*, 1987) was used to insert the *katG'::lacZ* fusion into chromosomal DNA by recombination in MC4100 carrying plasmid pAQ24 (*katG'::lacZ*) (Tartaglia *et al.*, 1989). The construction was carried out as described by Simons *et al.* (1987). Briefly, a culture of MC4100/pAQ24 was infected with a λRS45 lysate; recombination between λRS45 and the plasmids within the homologous *lacZYA-bla* region yielded a λ[Φ(*katG'::lacZ*)] fusion and a still incomplete *bla* gene. Recombinant phages were screened and identified by their Lac⁺ phenotype (blue plaques on LB agar supplemented with 40 μg/ml 5-bromo-4-chloro-3-indol-β-D-galactopyranose). A plaque-purified isolate, λAQ24, was integrated into the *att*λ site in the chromosome of the Lac⁻ strains RK4936 and TA4112. The resulting lysogens, BGF931 and BGF933, were identified by their Lac⁺ Amp^s phenotype.

For growth, strains were inoculated into LB broth (Miller, 1992) containing the appropriate antibiotic and incubated at 37 °C for 12–16 h with gentle shaking (200 rpm). The saturated cultures were diluted 100-fold into fresh LB and incubated for the indicated times at 37 °C with shaking at 200 rpm in flasks of volume 10–20-fold greater than the culture. Antibiotics were used at the following concentrations (in μg/ml): ampicillin, 100; tetracyclin, 12.5; kanamycin, 50; and chloramphenicol, 25.

Hydrogen Peroxide and Superoxide Anion Measurements—Intracellular concentrations of H_2O_2 and H_2O_2 production rates were measured as described previously (González-Flecha and Demple, 1984) by the horseradish peroxidase-scopoletin method (Boveris, 1994). Antimycin and rotenone, inhibitors of electron transport, and FCCP, an uncoupler, were used at final concentrations of 1, 0.5, and 0.2 μM, respectively. Bacterial suspensions (10^6 cells ml^{-1} in phosphate-buffered saline (PBS)) were incubated with the indicated compound for 10 min at 0–4 °C, washed twice with fresh PBS, and assayed as described (González-Flecha and Demple, 1994). The rates of H_2O_2 production were expressed as μM/s by assuming a cellular volume of 3.2×10^{-15} liter (Imlay and Fridovich, 1991a). Superoxide anion production was measured in isolated membranes prepared by a standard procedure (Imlay and Fridovich, 1991a). The rate of O_2^- production was measured by following the superoxide dismutase-sensitive rate of cytochrome *c* reduction at 550 nm ($\epsilon_{cytc}^{2+} - \epsilon_{cytc}^{3+} = 21\ mM^{-1}\ cm^{-1}$) (Boveris, 1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.4), 20 μM cytochrome *c*, 100 μM NADH, and ~0.2 mg/ml of membrane protein, with or without 50 units of bovine copper-zinc superoxide dismutase.

Enzymatic Activities—β-Galactosidase activity in sodium dodecyl sulfate-$CHCl_3$-treated cells was determined as described by Miller (1992). Two different approaches were used to assay the total catalase concentration of the cells. Catalase activity in cell lysates was determined as described previously (González-Flecha and Demple, 1994) and normalized to either the protein content of the lysate or the number of cells extracted. Protein concentration in the extracts was measured by the method of Lowry (Lowry *et al.*, 1951) using bovine serum albumin as standard. All the measurements were carried out in a Perkin-Elmer Lambda 3A UV/Vis spectrophotometer. In the second approach, catalase activity was measured by following the rate of elimination of H_2O_2 by cells suspended in PBS containing 2 mM H_2O_2. The H_2O_2 concentrations in the supernatants were determined by the horseradish peroxidase-scopoletin method. NADH-cytochrome *c* reductase activity was measured in bacterial membrane preparations (0.3–0.5 mg protein/ml) by following the superoxide dismutase-insensitive cytochrome *c* reduction as described above but using 50 μM cytochrome *c* in the presence of 50 units of bovine copper-zinc superoxide dismutase (Trumpower and Simmons, 1979).

Hydrogen Peroxide and Glucose/Glucose Oxidase Treatments—Overnight cultures of BGF931 and BGF933 were diluted 1:100 in fresh LB and grown for 1, 3, and 7 h. For the pulse-type treatment with H_2O_2, cultures at the indicated times were treated with different concentrations of H_2O_2, and the β-galactosidase activity directed by *katG'::lacZ* was followed for 30 min after addition of H_2O_2. In the case of treatment with glucose/glucose oxidase to generate a continuous flux of H_2O_2, cultures at the indicated times were supplemented with 10 mM glucose, and various amounts of glucose oxidase, and β-galactosidase activity were followed for 60 min after addition of glucose oxidase.

Statistics—Results are indicated as the mean value of four independent experiments ± S.E. Statistical significance of differences was analyzed by ANOVA, followed by Dunnett's test for other comparisons (Winer, 1971).

RESULTS

Hydrogen Peroxide Generation and Elimination in E. coli—The metabolic production of hydrogen peroxide in intact bacteria was evaluated initially in exponentially growing AB1157 after suspension of the cells in fresh PBS free of H_2O_2. The H_2O_2 concentration measured in the extracellular medium increased with time, reaching a plateau at 0.15 ± 0.01 μM after about 5 min for a cell density of 10^7 cells ml^{-1} (Fig. 1*A*). Bacteria suspended at 10- or 100-fold lower densities approached the same plateau, but with slower kinetics (Fig. 1*A*). Cells resuspended in PBS initially containing 1.5 μM H_2O_2 rapidly destroyed the extracellular H_2O_2 and again reached a plateau concentration of ~0.15 μM (Fig. 1*B*). The combined results correspond to the equilibration between intra- and extracellular H_2O_2 and indicate an intracellular steady-state concentration of H_2O_2 of 0.15 μM in exponentially growing *E. coli*.

Intracellular Sources of Hydrogen Peroxide—The contribution of cytosolic enzymes to the total production of H_2O_2 in intact *E. coli* was estimated by using the uncoupler of electron transport FCCP, which reduces to negligible levels the rate of H_2O_2 production associated with mitochondrial respiratory chain (Boveris and Chance, 1973). Addition of FCCP to *E. coli* AB1157 decreased the H_2O_2 steady-state concentration and production rate to about one-seventh the value of untreated cells (Table II). Thus, the production of oxygen free radicals by

[1] The abbreviations used are: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; LB, Luria-Bertani medium; PBS, phosphate-buffered saline; FMNH', flavin-semiquinone of the NADH dehydrogenase; UQH', ubisemiquinone; GO, glucose oxidase.

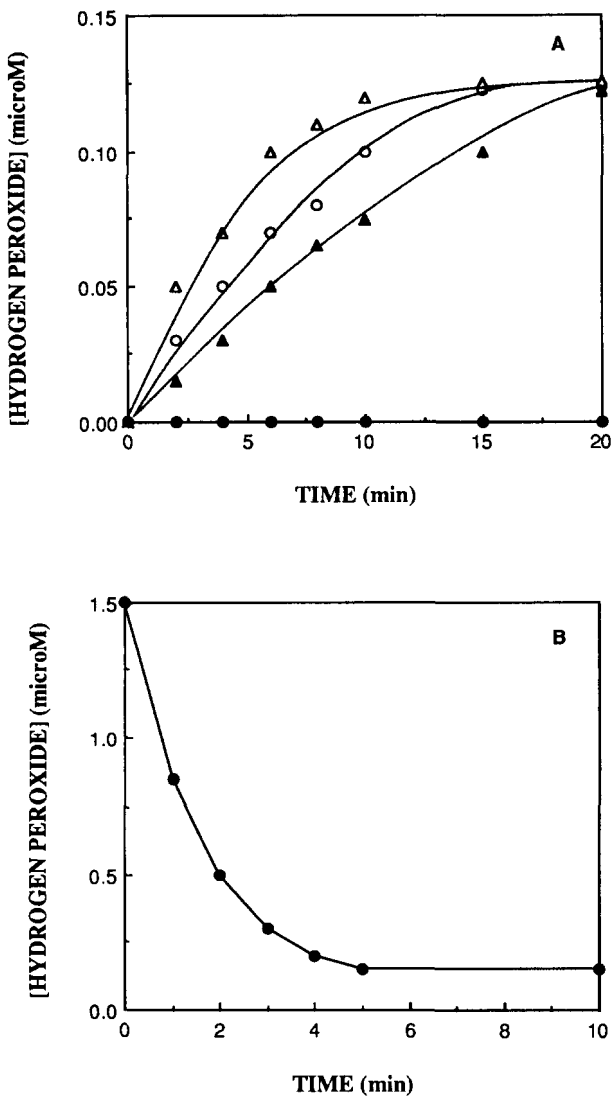


FIG. 1. **Hydrogen peroxide production and elimination by *E. coli*.** H_2O_2 concentration was measured in the extracellular medium of AB1157 incubated in PBS supplemented with 0 (*A*) and 1.5 μM (*B*) H_2O_2. *A*, cell densities after resuspension were 10^7 cells ml^{-1} (*open triangles*), 10^6 cells ml^{-1} (*open circles*), 10^5 cells ml^{-1} (*filled triangles*), and no cells (*filled circles*). *B*, cell density after resuspension was 10^6 cells ml^{-1}.

TABLE II

Effect of respiratory chain blockers on H_2O_2 production in intact E. coli and O_2^- production in membrane preparations

Exponentially growing *E. coli* AB1157 cells were incubated with FCCP, rotenone, or antimycin for 10 min, washed, resuspended in PBS, and assayed for H_2O_2 or O_2^- production and catalase activity. ND, not determined.

Treatment	$[H_2O_2]$	[Catalase]	$d[H_2O_2]/dt$	$d[O_2^-]/dt$
	μM	*pmol/mg protein*	μM/s	μM/s
None	0.15 ± 0.01	0.4 ± 0.1	1.4 ± 0.1	3.7 ± 0.5
FCCP	0.02 ± 0.01	0.4 ± 0.1	0.20 ± 0.05	ND
Rotenone	0.36 ± 0.05	0.4 ± 0.1	3.3 ± 0.3	5.1 ± 0.6
Antimycin	0.47 ± 0.04	0.4 ± 0.1	4.4 ± 0.5	8.0 ± 0.9

the respiratory chain in intact bacteria accounts for most of the H_2O_2 generation.

To identify specific sites of the respiratory chain at which single electrons might leak to form O_2^-, inhibitors of electron transport were used. The maximal rate of O_2^- production by autoxidation of the flavin-semiquinone of NADH-dehydrogenase ($FMNH^\cdot$) was measured by supplementing *E. coli* membrane preparations with NADH and the site I inhibitor rotenone (Boveris and Chance, 1973). Similarly, the maximal production of O_2^- by ubisemiquinone ($UQH^\cdot$) autoxidation was measured by supplementing *E. coli* membranes with NADH and the site II inhibitor antimycin (Boveris and Chance, 1973). The latter rate was assumed to represent the sum of the rates of O_2^- production at $UQH^\cdot$ plus $FMNH^\cdot$. The effect of these inhibitors on O_2^- and H_2O_2 production was also tested for intact bacteria by following the generation of H_2O_2 by the cells (Table II). As described previously for mitochondria (Turrens and Boveris, 1980), both rotenone and antimycin showed a biphasic effect on H_2O_2 production peaking at a concentration of 0.5 μM for rotenone and 1 μM for antimycin, when each compound blocked ~50% of the electron flux through the respiratory chain, as evaluated by the NADH-cytochrome *c* reductase activity (data not shown). Using these concentrations, both the rate of O_2^- production in *E. coli* membranes and the rate of H_2O_2 production in intact bacteria were increased 1.5–2-fold after rotenone treatment, and 2–3-fold after antimycin treatment (Table II). These results indicate that both NADH dehydrogenase and ubiquinone have significant potential to leak electrons to form O_2^- in *E. coli*.

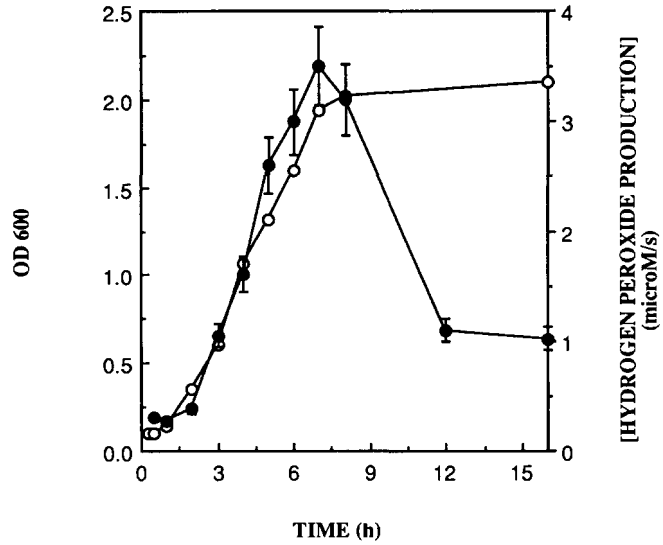


FIG. 2. **Hydrogen peroxide production during aerobic growth.** Overnight cultures of *E. coli* AB1157 were diluted 1:100 in fresh LB and incubated at 37 °C. At different time points samples were taken to measure OD_{600} (*open circles*) and assay H_2O_2 production rate (*filled circles*).

Hydrogen Peroxide and Superoxide Production during Aerobic Growth—The rate of H_2O_2 production during aerobic growth in rich medium showed a biphasic profile with a progressive increase during exponential growth, followed by a decrease after cessation of cell growth (Fig. 2). To estimate the contribution of oxygen free radicals produced at the respiratory chain level to the total oxygen free radical production, we studied the relationship between the rate of O_2^- production and the number of active respiratory chains. The O_2^- production by isolated membranes was linearly related to the number of respiratory chain units/cell, estimated by the NADH-cytochrome *c* reductase activity (Fig. 3).

Effect of Respiratory Chain Mutations on Intracellular H_2O_2 Generation—It has been reported that mutants defective in the biosynthesis of ubiquinone or menaquinone are also defective in the ability to induce the synthesis of catalases during aerobic growth (Hassan and Fridovich, 1978). To test the consequences of specific defects in NADH dehydrogenase or cytochrome oxidase on the production of H_2O_2 by *E. coli*, a series of isogenic

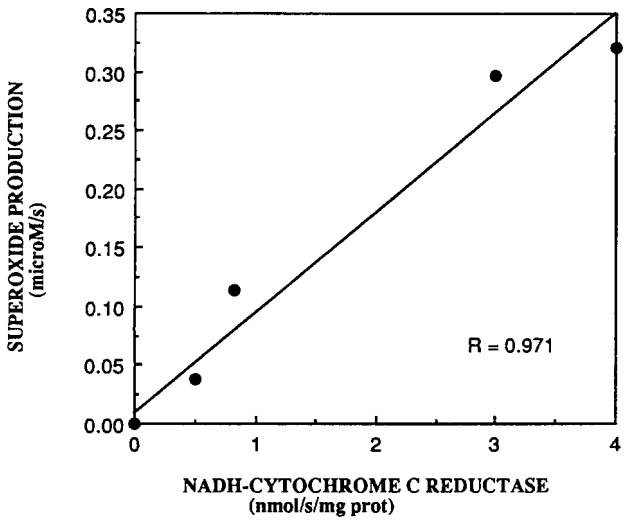


FIG. 3. **Superoxide anion production as a function of the number of respiratory chain units in isolated membranes.**

strains with mutations affecting these components was assayed for H_2O_2 steady-state and catalase concentrations. The rate of H_2O_2 production by GO103 (deficient in cytochrome oxidase *d*) was significantly lower than the rate of H_2O_2 production by either GO104 (deficient in cytochrome oxidase *o*) or the parental strain GR70N (Table III). The production of H_2O_2 was also significantly lower in strains MWC215 (deficient only in NADH dehydrogenase 2) and MWC232 (deficient in NADH dehydrogenase 1 and 2), and significantly higher in strain MWC190 (deficient in NADH dehydrogenase 1), compared with the control strain (Table III). In all the strains studied, the catalase concentration varied directly with the rate of H_2O_2 production. This balance kept the steady-state concentration of H_2O_2 within a 2-fold range for all the strains (Table III) and reflects a kind of homeostasis for reactive oxygen.

Growth Phase-dependent Triggering of a Response to H_2O_2—The above results suggested an almost continuous response of *E. coli* to different physiological rates of H_2O_2 generation. We therefore used a strain (BGF931) carrying a *katG'::lacZ* operon fusion to examine whether such a continuous response could be observed at the level of transcription of the catalase gene. We first examined the response of the reporter fusion to acute "pulse-type" exposures. The initial H_2O_2 concentrations required for maximal induction of *oxyR*-dependent *katG* transcription varied with the growth phase and were, respectively, ~15, ~25, and ~100 μM for 1-h, 2-h, and 3-h cultures (Fig. 4). The 7-h culture had a somewhat higher initial expression of *katG'::lacZ* but displayed a < 2-fold induction by H_2O_2 over the range 2 μM to 1 mM (Fig. 4*D*). These H_2O_2 concentrations provided about the same H_2O_2:catalase initial ratio for the 1–3-h cultures (Table IV), so that the H_2O_2 concentration remained above the physiological value for ~15 min (data not shown).

To evaluate regulation of *katG* expression in cells subjected to a "ramp-type" stimulus, we subjected *E. coli* BGF931 to a constant and defined external flux of H_2O_2 provided by glucose/glucose oxidase. The rates of H_2O_2 production were chosen from Fig. 2 to yield 2-fold increases in both the rate of production and the intracellular concentration of H_2O_2 at any given time. A 2-fold increase was chosen to mimic the elevated H_2O_2 generation observed between hours 2 and 3 of the growth curve (Fig. 2), which is associated with the induction of *katG* during exponential growth. As in the case of the pulse-type stimuli, the effective concentration of H_2O_2 depended on the catalase concentration in the cells, and on the growth phase. Catalase

TABLE III
Hydrogen peroxide metabolism in respiratory chain mutants

Exponentially growing bacteria were assayed for H_2O_2 and catalase concentrations. Cyt d^- and Cyt bo^-, deficient in cytochrome oxidases *d* and *o*, respectively; NDH-1^- and NDH-2^-, deficient in NADH dehydrogenases 1 and 2, respectively.

Strain (defect)	Steady state $[H_2O_2]$	[Catalase]	$d[H_2O_2]/dt$
	μM	*pmol/mg protein*	*μM/s*
GR70N (parental)	0.12 ± 0.02	0.5 ± 0.1	1.40 ± 0.05
GO103 (Cyt d^-)	0.09 ± 0.01	0.26 ± 0.08	0.54 ± 0.02[a]
GO104 (Cyt bo^-)	0.09 ± 0.03	0.7 ± 0.1	1.50 ± 0.05
MWC215 (NDH-2^-)	0.14 ± 0.03	0.30 ± 0.04	1.10 ± 0.02[a]
MWC190 (NDH-1^-)	0.15 ± 0.03	0.3 ± 0.1	1.0 ± 0.1[a]
MWC232 (NDH-1^-, NDH-2^-)	0.17 ± 0.03	0.3 ± 0.1	1.2 ± 0.05[a]

[a] Significant difference compared with the wild type strain with $p < 0.005$.

activity in lag-phase cells destroyed only 18% of the H_2O_2 produced by glucose/glucose oxidase (Fig. 5*A*). In exponentially growing cells 90% of the external flux was destroyed by intracellular catalase (Fig. 5*B*). Despite this difference, the 2-fold increases in H_2O_2 production significantly induced *katG'::lacZ* expression during both the lag and the exponential phase (Fig. 6). In contrast, *katG'::lacZ* was hardly induced during early stationary phase by the 2-fold increase in the H_2O_2 production rate (Fig. 6).

DISCUSSION

The experiments presented here indicate that the major source of H_2O_2 in intact *E. coli* is probably the respiratory chain, which can account for as much as ~87% of the total H_2O_2 production. Similar to eukaryotic mitochondria, the leakage of single electrons from the bacterial respiratory chain was observed at the NADH dehydrogenase and ubiquinone sites. Accordingly, changes both in the number of respiratory chain units/cell and in the composition of the electron transport chain affected the rate of hydrogen peroxide production. Our results showed that the rate of H_2O_2 production changes dramatically during aerobic growth and was linearly related to the number of respiratory chain units/cell estimated by the specific activity of NADH-cytochrome *c* reductase. Bacteria seem to cope with this changing generation of oxygen radicals by elevating the expression of antioxidant functions, represented here by the *katG*-encoded catalase. Indeed, mutational suppression of catalase-hydroperoxidase I, but not of catalase-hydroperoxidase II, increased intracellular H_2O_2 concentrations to ~0.3 μM, which demonstrates a role for catalase-hydroperoxidase I as a defensive enzyme.[2]

Interestingly, the calculated rate of H_2O_2 production in exponentially growing *E. coli* approached values similar to the rate of production in mammalian cells (~4 μM s^{-1}) (González-Flecha *et al.*, 1993). This production followed the theoretical 2:1 stoichiometry for superoxide:hydrogen peroxide ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), which indicated that most of the H_2O_2 generation in *E. coli* arises as a by-product of O_2^- generation.

Mutational inactivation of different components of the respiratory chain also affected the rate of H_2O_2 production. Elimination of either or both NADH dehydrogenases (in strains MWC190, MWC215, or MWC232) decreased the rate of H_2O_2 production. NADH dehydrogenase 1 is a multisubunit complex homologous in structure and function to the eukaryotic complex I (Meinhardt *et al.*, 1989). This enzyme contains four iron-sulfur clusters and FMN and couples its reaction to the

[2] B. González-Flecha and B. Demple, manuscript in preparation.

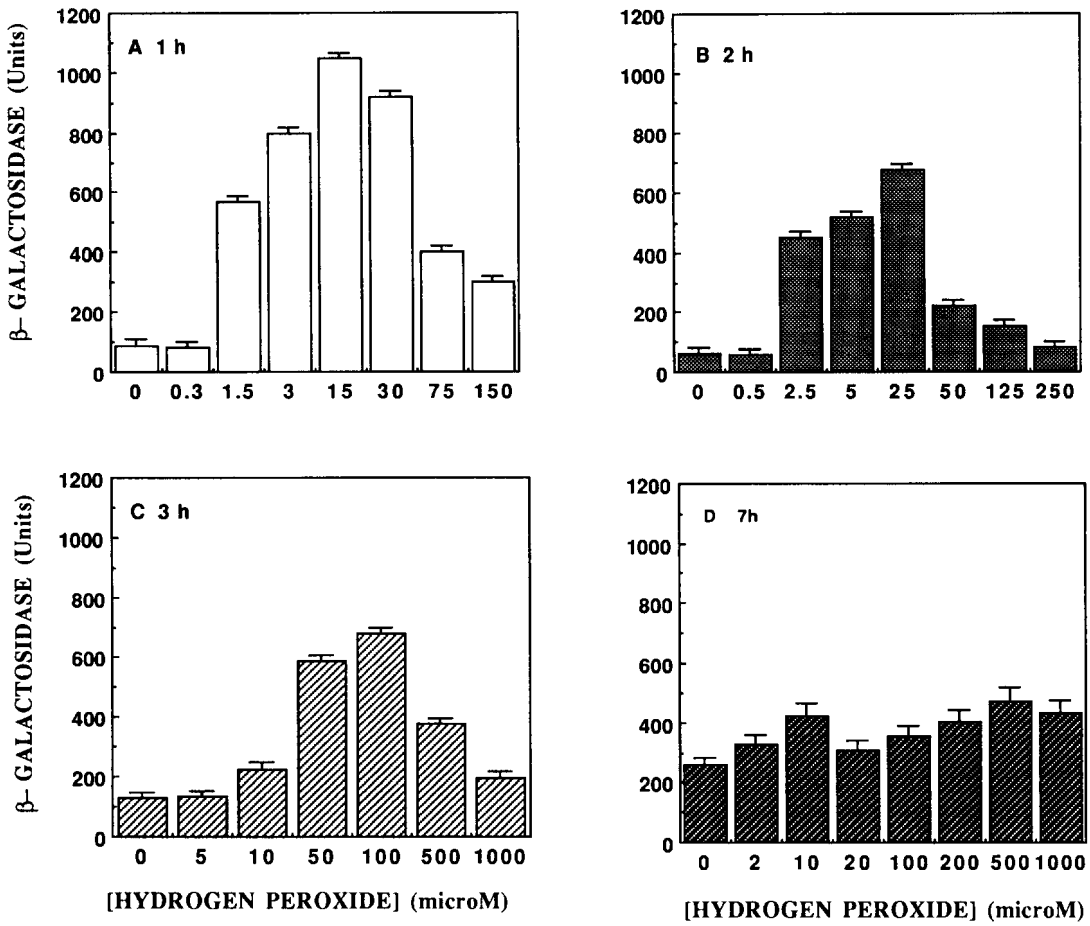


FIG. 4. **Induction of katG'::lacZ by acute H_2O_2 exposure.** Overnight cultures of BGF931 in LB were diluted 1:100 in fresh LB and incubated at 37 °C for 1, 2, 3, or 7 h. At the indicated times, samples were treated with the indicated concentration of H_2O_2 and incubated for a further 30 min. At 5, 10, 20, and 30 min, samples were taken and assayed for β-galactosidase activity. No increase in β-galactosidase activity was observed in the Δ*oxyR* strain BGF933 (not shown). Values in the figure correspond to maximal induction, obtained 10 min after H_2O_2 addition.

TABLE IV

Elimination of H_2O_2 by intracellular catalase in pulse-type models of oxidative stress

Overnight cultures of BGF931 in LB were diluted 1:100 in fresh LB and incubated at 37 °C. Cultures grown for 1, 2, and 3 h were treated with the concentration of H_2O_2 required for maximal induction of the *katG'::lacZ* fusion, and the concentration of H_2O_2 in the extracellular medium was followed. $[H_2O_2]_i$, initial concentration of H_2O_2; elimination $t_{1/2}$, half-life of H_2O_2 in the extracellular medium; $[Catalase]_i$, catalase concentration before the addition of H_2O_2

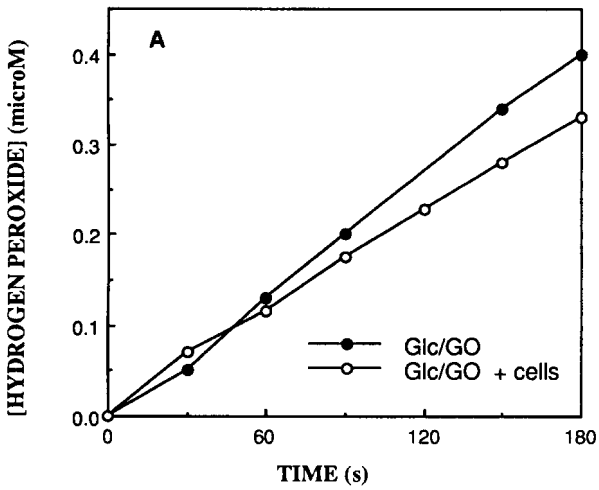
Culture time (h)	$[H_2O_2]_i$	Elimination $t_{1/2}$	$[Catalase]_i$	H_2O_2/catalase[a]
	μM	*min*	*fmol/10⁶ cells*	
1	15	9.0	8.8	18,000
2	25	7.5	6.2	16,000
3	100	2.5	17.6	13,000

[a] This number is the ratio of H_2O_2 molecules to catalase molecules per cell at time 0.

generation of proton-motive force (Matsushita *et al.*, 1987). NADH dehydrogenase 2, in contrast, contains FAD and no iron (Hayashi *et al.*, 1989), and its function is not related to proton translocation (Matsushita *et al.*, 1987). Both dehydrogenases would be likely to react with O_2 to generate superoxide. The results presented here show that the inactivation of either NADH dehydrogenase 1 or NADH dehydrogenase 2 decreased the rate of H_2O_2 production by only 25–30%. These results contrast with those of Imlay and Fridovich (1991b), in which the residual O_2^- production in membranes isolated from a strain lacking NADH dehydrogenase 2 was only 8% of that for the parental strain. However, in intact bacteria lacking the NADH dehydrogenases, reducing equivalents can and evidently do still enter the respiratory pathway at the ubiquinone level via alternative dehydrogenases, such as succinate dehydrogenase or lactate dehydrogenase (Ingledew and Poole, 1984).

H_2O_2 production was also affected by changing the energy status of *E. coli* by directing the electron flux through components with higher or lower energetic efficiency. In isolated mitochondria H_2O_2 production strongly depends upon the energy status; an "energized" condition (state 4, with slow O_2 consumption and ADP phosphorylation) corresponds to a highly reduced steady state for the respiratory carriers and to a relatively high H_2O_2 generation; a "de-energized" condition (state 3, with fast O_2 consumption and ATP production) corresponds to a highly oxidized steady state for the respiratory chain components and to a relatively low H_2O_2 production (Boveris and Chance, 1973). In our experiments, mutants utilizing the so-called "coupled" components of the respiratory chain (NADH dehydrogenase 1 in strain MWC215 and cytochrome *bo* in strain GO103) (Calhoun *et al.*, 1993) appear to be in a de-energized condition, with the respiratory carriers largely in the oxidized state and generating H_2O_2 at a relatively low rate. In contrast, the mutant strain GO104 utilizing the "uncoupled" cytochrome *d* (Calhoun *et al.*, 1993) seems to be in an energized state with highly reduced electron transport carriers. This observation could extend to the growth of bacteria on different carbon sources or other conditions that determine different patterns of "coupled-uncoupled" components.

Mutational changes in the composition of the respiratory



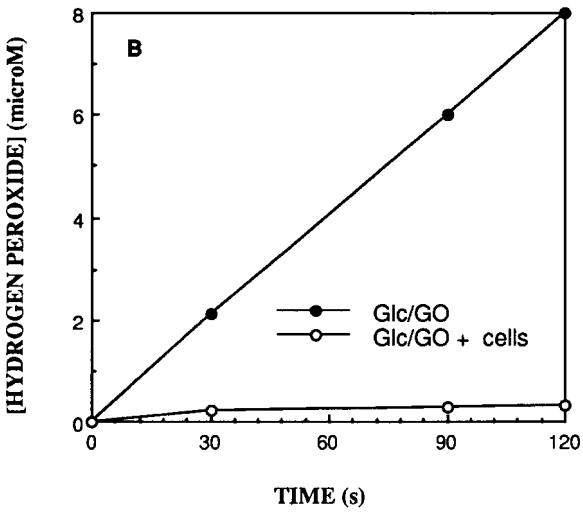


FIG. 5. **Time course of H_2O_2 production by the glucose/glucose oxidase system.** Growth conditions were as in Fig. 4. At 1 h (*A*) and 3 h (*B*), glucose (*Glc*) was added to a final concentration of 10 mM and followed by 0.4 mg/ml (*A*) or 10 mg/ml (*B*) of glucose oxidase (*GO*) to generate H_2O_2 at a flux equal to the intracellular rate (Fig. 2). The amount of H_2O_2 in the culture medium was measured at the indicated times.

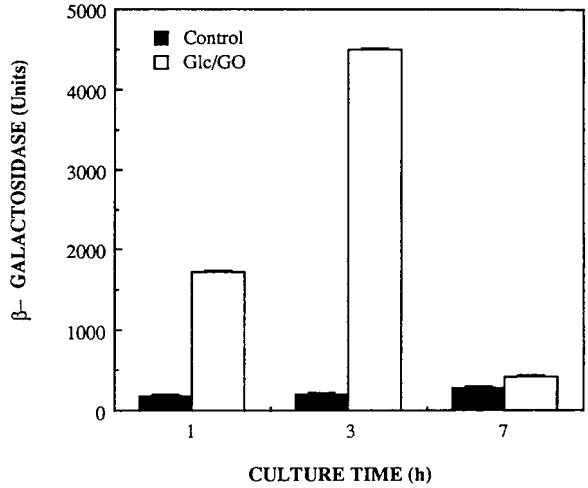


FIG. 6. **Induction of *katG* expression by extracellular fluxes of H_2O_2.Experimental conditions were as described in Fig. 5.** Values in the figure correspond to maximal induction, obtained at 15, 45, and 60 min after glucose oxidase (*GO*) addition to the 1-h (0.4 mg/ml), 3-h (10 mg/ml), and 7-h (60 mg/ml) cultures, respectively. No increase in β-galactosidase activity was observed in BGF933 (not shown).

chain prompted complementary changes in catalase activity. It is worth noting that, even in the strains with a decreased rate of H_2O_2 production (GO103, MWC215, and MWC232), catalase expression was regulated to keep the intracellular H_2O_2 steady-state concentration at 0.1–0.2 μM. These results suggest that at least some basal induction of the *oxyR* regulon occurs even without exogenous H_2O_2 stress, triggered by values that exceed the 0.1–0.2 μM physiological value. This represents a sensitivity ≥25-fold greater than previously associated with *oxyR*-dependent catalase induction, which occurred in response to a pulse-type exposure to 5 μM H_2O_2 (Demple and Halbrook, 1983). A key difference is likely to be the more constant H_2O_2 generation by respiration leakage, as in the case of the ramp-type oxidative stress, which might exert a cumulative effect by constantly activating OxyR protein. We have shown here that pulse-type stimuli may provide an increased H_2O_2 concentration for only ~15 min. A critical question for understanding the difference between these two situations is the half-life of activated OxyR protein, which has not been determined. A long half-life would favor more dramatic inducing effects of a modest H_2O_2 pulse (1–5 μM) than would a short half-life. Important factors that could remove activated OxyR might be proteolysis and direct reversal of the activated form, which is thought to be an oxidized protein (Storz *et al.*, 1990).

It is interesting to note that the magnitude of the H_2O_2 response not only depends on the magnitude and type of stimulus but also on the growth phase, being maximal at logarithmic phase and almost negligible during early stationary phase. This observation is in good agreement with the reported resistance of bacteria to H_2O_2 in stationary phase cultures (Jenkins *et al.*, 1988; Hengge-Aronis. 1993) and with the dual regulation of *katG* (Ivanova *et al.*, 1994) and *dps* and *oxyS* (Altuvia *et al.*, 1994) by both *oxyR* and by the stationary phase regulator *rpoS*. We do not know if *rpoS* regulation merely supersedes that by *oxyR* in stationary phase cells, or whether the activity of OxyR may be less under those conditions.

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