JOURNAL OF BACTERIOLOGY, July 1987, p. 2967–2976
0021-9193/87/072967-10$02.00/0

Vol. 169, No. 7

Mutagenesis and Stress Responses Induced in *Escherichia coli* by Hydrogen Peroxide

JAMES A. IMLAY AND STUART LINN*

Department of Biochemistry, University of California, Berkeley, California 94720

Received 29 December 1986/Accepted 31 March 1987

Killing of *Escherichia coli* by hydrogen peroxide proceeds by two modes. Mode one killing appears to be due to DNA damage, has a maximum near 1 to 3 mM H_2O_2, and requires active metabolism during exposure. Mode two killing is due to uncharacterized damage, occurs in the absence of metabolism, and exhibits a classical multiple-order dose-response curve up to at least 50 mM H_2O_2 (J. A. Imlay and S. Linn, J. Bacteriol. 166:519–527, 1986). H_2O_2 induces the SOS response in proportion to the degree of killing by the mode one pathway, i.e., induction is maximal after exposure to 1 to 3 mM H_2O_2. Mutant strains that cannot induce the SOS regulon are hypersensitive to peroxide. Analysis of the sensitivities of mutants that are deficient in individual SOS-regulated functions suggested that the SOS-mediated protection is due to the enhanced synthesis of recA protein, which is rate limiting for recombinational DNA repair. Specifically, strains wholly blocked in both SOS induction and DNA recombination were no more sensitive than mutants that are blocked in only one of these two functions, and strains carrying mutations in *uvrA*, *-B*, *-C*, or *-D*, *sfiA*, *umuC* or *-D*, *ssb*, or *dinA*, *-B*, *-D*, *-F*, *-G*, *-H*, *-I*, or *-J* were not abnormally sensitive to killing by H_2O_2. After exposure to H_2O_2, mutagenesis and filamentation also occurred with the dose response characteristic of SOS induction and mode one killing, but these responses were not dependent on the *lexA*-regulated *umuC* mutagenesis or *sfiA* filamentation functions, respectively. Exposure of *E. coli* to H_2O_2 also resulted in the induction of functions under control of the *oxyR* regulon that enhance the scavenging of active oxygen species, thereby reducing the sensitivity to H_2O_2. Catalase levels increased 10-fold during this induction, and *katE katG* mutants, which totally lack catalase, while not abnormally sensitive to killing by H_2O_2 in the naive state, did not exhibit the induced protective response. Protection equal to that observed during *oxyR* induction could be achieved by the addition of catalase to cultures of naive cells in an amount equivalent to that induced by the *oxyR* response. Thus, the induction of catalase is necessary and sufficient for the observed *oxyR*-directed resistance to killing by H_2O_2. Although superoxide dismutase appeared to be uninvolved in this enhanced protective response, *sodA sodB* mutants, which totally lack superoxide dismutase, were especially sensitive to mode one killing by H_2O_2 in the naive state. *gshB* mutants, which lack glutathione, were not abnormally sensitive to killing by H_2O_2.

Killing of *Escherichia coli* by hydrogen peroxide results from damage that is produced by at least two pathways which are distinguishable by metabolic, kinetic, and genetic criteria (23). Metabolically inactive cells are subject to a single mode of killing, mode two, which exhibits a broad shoulder of resistance to H_2O_2. Metabolically active cells exhibit an additional mode of killing, mode one, characterized by its greater rate of killing at low (ca. 1 to 3 mM) versus intermediate (ca. 10 mM) concentrations of H_2O_2 (23). The location of lethal cell damage has not been identified for mode two killing, but mode one killing appears to result from DNA damage, since strains deficient in RecA protein, exonuclease III, exonuclease V (RecBC enzyme), or DNA polymerase I are especially vulnerable to this mode of killing.

Exposure of *E. coli* to low concentrations of hydrogen peroxide induces a protective response which confers increased resistance to subsequent exposures (14). This response is governed by a regulatory locus, *oxyR*, which appears to encode a positive effector (12), and it includes an increased synthesis of catalase, alkyl hydroperoxide reductase, and other polypeptides (12).

The sensitivity of *recA* strains to mode one killing (23) suggests that H_2O_2 also activates the SOS response. We report here that exposure of *E. coli* to low concentrations of hydrogen peroxide does indeed induce the SOS response and that this response protects against mode one killing through an enhanced ability to carry out recombinational DNA repair. Conversely, the *oxyR* regulon exerts its protective effect primarily through an enhanced ability to scavenge partially reduced oxygen species. Thus, two stress responses are induced by H_2O_2 that act by nonoverlapping processes to protect against lethal doses of H_2O_2.

MATERIALS AND METHODS

The bacterial strains which were utilized in this study are listed in Table 1. Hydrogen peroxide was purchased as a 30% aqueous solution from Mallinckrodt, Inc. (St. Louis, Mo.); beef liver catalase was a 20-mg/ml, 65,000-U/mg suspension from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); chloramphenicol, trimethoprim, tetracycline, xanthine, cytochrome *c*, and xanthine oxidase were from Sigma Chemical Co. (St. Louis, Mo.).

Liquid cultures were grown in K medium (1% glucose, 1% Casamino Acids [Difco Laboratories, Detroit, Mich.], 1 μg of thiamine hydrochloride per ml, 1 mM $Mg SO_4 \cdot 7H_2O$, 0.1 mM $CaCl_2$, M9 salts [36]); cultures were grown with vigorous shaking. Cells were challenged with H_2O_2 at a density of 1×10^7 to 4×10^7 CFU/ml in 1 ml of K medium for 15 min at 37°C. The challenge was terminated either by dilution into

* Corresponding author.

TABLE 1. 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*	1
AB3049	F⁻ *endA thyA deoA* or *B gal xyl thi*	P. Howard-Flanders
AB3062	As AB3049 plus *uvrB5*	P. Howard-Flanders
BW9091	As AB1157 plus *xth-1*	35
BW9116	Hfr KL16 PO-45 Δ(*xth-pncA*)*90 zdh-201*::Tn*10 relA1 spoT1 thi-1*	B. Weiss
C600 (λ)	*thy leu sup*⁺	H. Echols
CSH7	*lacY rpsL thi-1*	30
DM2572	*recA430 srlC300*::Tn*10 lexA51 supD*	H. Echols
DM49	As AB1157 plus *lexA3*	39
GC4540	*thr leu his ura str tif tsl sfiA*::Tn*5*	S. Gottesman
GW1000	*lac*Δ*U169 thr leu his arg ilv*(Ts) *gal str tif sfiA*	24
GW1010	As GW1000 plus *dinA*::Mu d	24
GW1030	As GW1000 plus *dinB1*::Mu d	24
GW1040	As GW1000 plus *dinD1*::Mu d	24
GW1070	As GW1000 plus *dinF1*::Mu d	24
GW2100	As AB1157 plus *umuC122*::Tn*5*	17
GW3703	As AB1157 plus *uvrD260*::Tn*5*	40
GW4750	As AB1157 plus *dinG1*::Mu d	G. Walker
GW4760	As AB1157 plus *dinH1*::Mu d	G. Walker
GW4770	As AB1157 plus *dinI1*::Mu d	G. Walker
GW4780	As AB1157 plus *dinJ1*::Mu d	G. Walker
JC14330	*trpA46* JM103::Δ(*lac-pro*) *thi strA supE endA hsdR* F′ *traD36 proAB lacI*ᵠ *Z*Δ*M15*	A. J. Clark
JC15430	As JC14330 plus *recF143*	A. J. Clark
JC15453	As JC14330 plus *recA730*	A. J. Clark
JC4583	F⁻ *gal his thi endA*	3
JC4588	As JC4583 plus *recA56*	3
JC6720	*gal endA recB recC*	A. J. Clark
JC6720 (λ)	As JC6720 plus λ lysogen	This paper
JC6721	*gal endA recC*	A. J. Clark
JC7623	As AB1157 plus *sbcB15 recB21 recC22*	J. Fuchs
JC8679	*thr-1 leu-6 thi-1 galK2 proA2 his-4 argE3 str-31 tsx-33 recB21 recC22 sbcA23 supE44*	F. W. Stahl
JC9239	As AB1157 plus *recF143*	A. J. Clark
JF511	As JC7623 plus *gshB*::Kan	J. Fuchs
JI110	As AB1157 plus *recA430 srlC300*::Tn*10*	This paper
JI112	As CSH7 plus *zdh-201*::Tn*10* Δ(*xth-pncA*)*90*	This paper
JI114	As UM1 plus *zdh-201*::Tn*10* Δ(*xth-pncA*)*90*	This paper
JI120	As JC14330 plus *recC*	This paper
JI121	As JC15430 plus *recC*	This paper
JI122	As JC15453 plus *recC*	This paper
JI130	As AB1157 plus (*sodA*::Mu d PR13)*25*	This paper
JI131	As AB1157 plus (*sodB-kan*)1-Δ2	This paper
JI132	As AB1157 plus (*sodA*::Mu d PR13)*25* (*sodB-kan*)1-Δ2	This paper
NH4905	As AB3049 plus *uvrA6*	P. Howard-Flanders
NH5132	As AB3049 plus *uvrC34*	P. Howard-Flanders
QC909	F⁻(*sodA*::Mu d PR13)25 ϕ(*sodB-kan*)1-Δ2 Δ*lacU169 rpsL*	18
SP648	W3110 plus *tyrA*::Tn*10*	R. Lloyd
SP649	W3110 plus *tyrA*::Tn*10 recN262*	R. Lloyd
UM1	As CSH7 plus *katE1 katG14*	30

M9 salts or by the addition of 2 μg of catalase. For survival studies, cells were plated in top agar onto L agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.1% glucose, 2.5 mM $CaCl_2$, 1% agar), and colonies were counted after 24 to 48 h. For studies of postchallenge growth or division, the H_2O_2 was eliminated by the addition of catalase, and filamentation on the surface of plates was visualized by light microscopy. For pretreatment to induce protective responses, H_2O_2 was present in a final concentration of 60 μM, and cells were incubated for an additional 70 min before the challenge.

Bacteriophage was inactivated by exposure of λ *vir* to 12.5 mM H_2O_2 in the presence of 4 μM $CuSO_4$ in M9 salts; at the times indicated, samples were removed into M9 salts buffer containing 25 U of catalase per ml. Phage inactivation by far-UV light was by irradiation in M9 salts on a watchglass with a model UVGL-25 Mineralight lamp (254 nm) at 1.4 J m^{-2} s^{-1} (Ultraviolet Products, Inc.).

Thymidylate synthetase (*thyA*) mutants were scored by plating on minimal agar plates (36) in 2 ml F-top agar (36) containing 2 mg of thymine and 0.2 mg of trimethoprim. Phage lambda induction was determined by plating challenged lysogens with approximately 2×10^7 CFU of the indicator strain, AB1157, and counting within 8 h. (Delayed counting results in a high background of spontaneous induction; coplating the lysogens with too many indicator bacteria obscures the delayed appearance of phage which are induced by high concentrations of H_2O_2.)

P1 transduction was as described previously (36). Strain JI110 was generated by transduction of *recA430* from DM2572 to AB1157; selection was for the linked tetracycline resistance, and cotransduction was confirmed by UV sensitivity. Strains JI120, JI121, and JI122 were generated by transduction of *recC thyA*⁺ from JC6721 into spontaneous trimethoprim-resistant (*thyA*) mutants of strains JC14330, JC15430, and JC15453, respectively. Selection was for *thy*⁺,

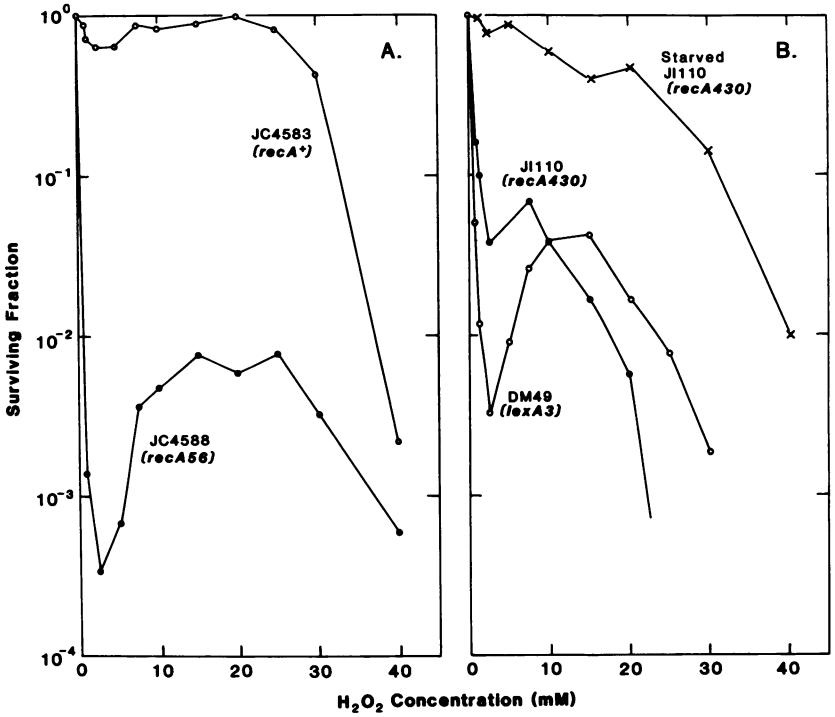

FIG. 1. Survival of cells with a defective SOS response after challenge with H_2O_2.

and transductants were screened by UV sensitivity. Strains JI112 and JI114 were generated by cotransduction of *xth* with *zdh-201*::Tn*10* from BW9116 into CSH7 and UMI, respectively, with selection for tetracycline resistance. Strains JI130, JI131, and JI132 were generated by consecutive transduction of *sodA*-linked chloramphenicol resistance and *sodB*-linked kanamycin resistance into AB1157. Transductants were screened with superoxide dismutase activity gels (4). Catalase assays (5) and superoxide dismutase assays (33) were as described previously. One unit of catalase degrades 1 μmol of H_2O_2 per min at 8 mM H_2O_2 and room temperature. Protein concentrations were determined by the method of Bradford (7).

RESULTS

Protection against mode one killing by SOS induction. RecA protein acts as a positive regulator of the SOS system: in response to blockage of the replication fork, it cleaves the LexA protein, a transcriptional repressor of the genes of the regulon (28). RecA protein is also an obligate participant in recombinational events (13), including those which repair DNA damage. Thus, the sensitivity of a *recA56* mutant to mode one killing by hydrogen peroxide which was previously observed (23) (see also Fig. 1A) could be due to its inability to induce SOS functions, its inability to carry out DNA recombination, or both.

These possibilities can be experimentally distinguished with the *recA430* allele, a mutation that yields a RecA protein that is competent for DNA recombination but defective in SOS induction by virtue of a reduced ability to cleave the LexA repressor (42). A strain carrying this allele was in fact sensitive to mode one killing by hydrogen peroxide (Fig. 1B), as indicated both by the shape of the killing curve and by the resistance conferred by starvation (Fig. 1B). Moreover, a *lexA3* strain, in which the SOS regulon is uninducible owing to mutation at the Ala-Gly cleavage site of the LexA repressor (31), was tested for sensitivity. This strain, too, was extremely sensitive to mode one killing (Fig. 1B). Together these data indicate that the ability of cells to survive normally after exposure to low concentrations of hydrogen peroxide is dependent on the induction of some SOS-regulated function(s). (The somewhat reduced sensitivity of the *recA430* strain relative to that bearing the *lexA3* mutation might be attributable to the low, but significant, level of residual SOS induction reported for *recA430* strains [15].)

Dose response for SOS induction by H_2O_2 mirrors the dose response for mode one killing. The in vivo activation of the RecA protease activity after exposure of *E. coli* to hydrogen peroxide can be monitored by the induction of lambda prophage, since activation of the RecA protease results in cleavage of the lambda cI repressor and hence derepression of phage lytic functions (43). Lambda induction after exposure to hydrogen peroxide exhibited the dose dependence which is characteristic of mode one killing (Fig. 2A): the induction frequency was maximal near 1 mM and fell to a lower, constant plateau level above 5 mM. Indeed, quantitative comparison of the dose dependencies of lambda induction in a wild-type strain and the rate of mode one killing in a DNA-repair-deficient strain showed remarkable equivalence (Fig. 2B). This equivalence might indicate that mode one killing lesions and the DNA damage that induces the SOS response arise from a common intermediate.

SOS-mediated protection does not appear to be due to enhanced DNA excision repair. Among the SOS-induced responses are increased efficacy of excision and recombinational DNA repair, enhanced mutagenesis, and cell filamentation (45). We attempted to determine which, if any, of these functions might be responsible for SOS-dependent protection against mode one killing.

The *polA1* allele confers extreme sensitivity to mode one

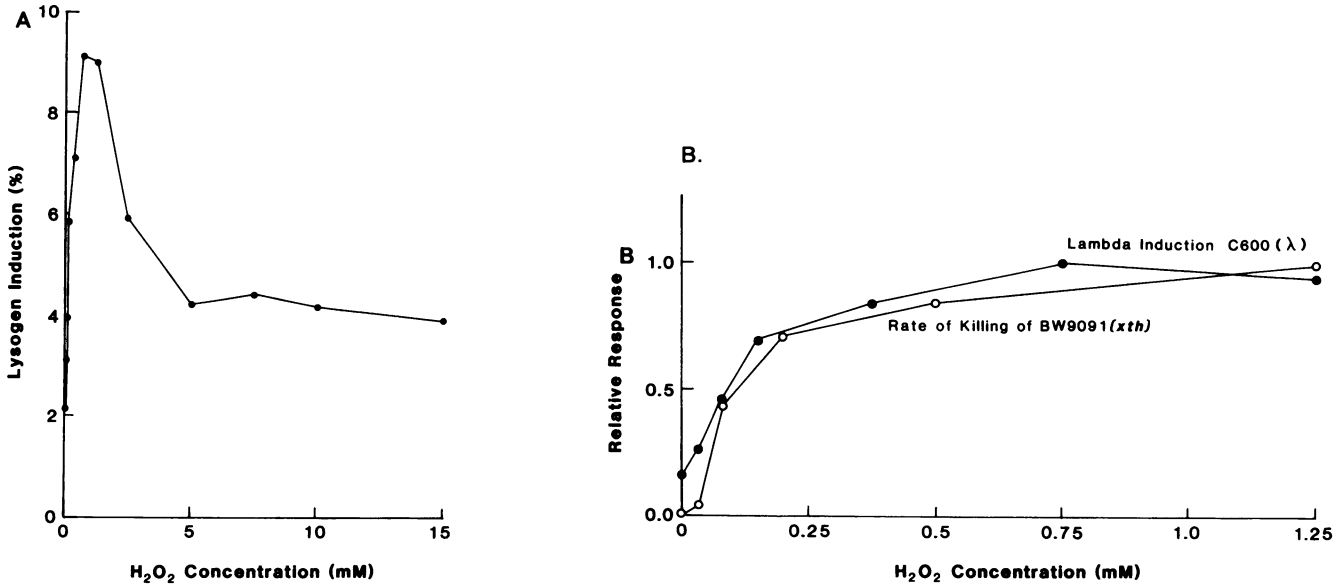

FIG. 2. Induction of lambda lysogens by H_2O_2. (A) Lysogens induced as a percentage of challenged cells. (B) Lysogen induction of C600 (λ) and rate of killing of BW9091 (*xth*) as a function of H_2O_2 concentration. Responses were normalized relative to the maximum observed: 9.1% for phage induction and 0.59 min^{-1} for killing.

killing (23). Since DNA polymerase I takes part in the *uvrABCD* nucleotide excision repair mechanism (25), and since the expression of these *uvr* genes is enhanced after SOS induction (45), it is conceivable that the *lexA3* and *polA1* sensitivities might both result from the absence of this repair pathway. However, *uvrA*, *uvrB*, *uvrC*, and *uvrD* mutations do not confer sensitivity to hydrogen peroxide (data not shown). Thus, the requirements for DNA polymerase I and for the SOS response for normal resistance appear to be unrelated to this pathway of nucleotide excision repair.

Peroxide-mediated mutagenesis and role of *umuDC*. When a dose response for H_2O_2-mediated mutagenesis was determined by scoring forward mutations in the thymidylate synthetase gene, a linear component was observed only above 10 mM H_2O_2 (Fig. 3). This first-order response above 10 mM H_2O_2 is analogous to the proportionality seen for mode two killing as a function of H_2O_2 concentration (23). Superimposed this linear response was a component at low doses with the unique shape of the mode one killing and lambda induction dose-response curves: a maximum response was observed at 2.5 mM H_2O_2, with a lower, dose-independent response at moderate doses (Fig. 3). It would therefore appear that the damage or damaging agents that result in mode one and mode two killing also have mutagenic components.

The similarity of the shapes of the dose-response curves for SOS induction and mutagenesis (compare Fig. 2A and 3) and the fact that the SOS response to UV irradiation is accompanied by enhanced mutagenesis suggest that the mutagenesis component at low H_2O_2 concentrations is dependent on SOS induction. Unfortunately, mutation frequency cannot be directly measured in the *lexA* or *recA* mutants that are defective in the induction of SOS response, because of the high degree of killing of these cells. However, mutagenesis induced by UV light via the SOS response (2) is dependent on the *umuDC* operon which is activated during SOS induction, but H_2O_2-mediated mutagenesis was unaffected by loss of *umuC* function (Fig. 3). (*umuC* and *umuD* mutants are not abnormally sensitive to killing by hydrogen peroxide [Fig. 3, inset].) In conclusion, the induction of *umuDC* during the SOS response does not protect against mode one killing; furthermore, while *umuDC* induction enhances mutagenesis elicited by UV, it does not have an apparent effect on that elicited by H_2O_2.

Exposure to H_2O_2 results in a filamentous response that does not require SOS induction. Peroxide-treated cells exhibit a delay in cell division after removal of the toxin. When this delay period was quantitated as the time required to achieve one population doubling after a peroxide challenge, two components were apparent in the dose-response curve (Fig. 4). One component increased linearly with peroxide concentration and was apparent with both metabolically active and inactive cells. A second component was observed only when metabolically active cells were exposed to H_2O_2. This component gave a submaximum at 2 to 3 mM H_2O_2 (Fig. 4).

Microscopic examination during the division delay period of bacteria that were challenged when metabolically active revealed that at all doses the delay is made up of two phases (which are schematized in Fig. 4). There is first a dose-proportionate period which is characterized by the total absence of cell growth—the cells remain unit sized. This growth lag is also observed with metabolically inactive cells which had been starved before and during the challenge (data not shown). Hence, this first period appears to be a consequence of the damage that is related to mode two killing, both because of the first-order dose-response and because of the lack of dependence on metabolic activity during challenge.

At all doses, the period of growth delay was followed by a period of filamentation during which the surviving cells grew, but did not septate. The period of filamentation was constant at roughly 40 min for challenges at intermediate and high doses of H_2O_2, but had the peculiar submaximum at low doses which is characteristic of mode one killing, mutagenesis, and lambda induction (Fig. 4). (Starved cells do not exhibit a filamentous response, nor do they have the

submaximum [data not shown].) The shape of the dose-response curve for filamentation and the requirement for metabolism suggest that the filamentous response is required specifically for the repair of DNA lesions which give rise to mode one killing. In fact, cells which are ultimately killed by the mode one mechanism enter into a filamentous response but never restore septation (23). In summary, repair of peroxide-induced damage appears to be sequential: mode two killing lesions are repaired during a period of growth delay, and then mode one killing lesions are repaired during a period of filamentation.

Filamentation occurs also as a consequence of the SOS response to UV light and is generally mediated via derepression of the *sfiA* gene (22). However, *sfiA* strains as well as SOS-deficient *recA* strains gave the normal filamentous response after exposure to hydrogen peroxide. Moreover, *sfiA* mutations did not confer unusual sensitivity to killing by H_2O_2.

Mu d::*lac* insertions into *din* genes do not sensitize cells to hydrogen peroxide. Using the Mu d::*lac* fusion method of Casadaban and Cohen (11), Kenyon and Walker (24) have identified genes which are under *recA-lexA* regulation, many of which have yet to be associated with a biochemical acitivity. Because these *din* (damage-inducible) mutants are generated by an operon-fusion technique, the function of the gene is likely to be disrupted. When the peroxide sensitivity of the eight available *din* mutants (*dinA*, *dinB*, *dinD*, *dinF*, *dinG*, *dinH*, *dinI*, and *dinJ*) was evaluated, none of these was particularly sensitive to mode one killing by 2.5 mM H_2O_2 (data not shown).

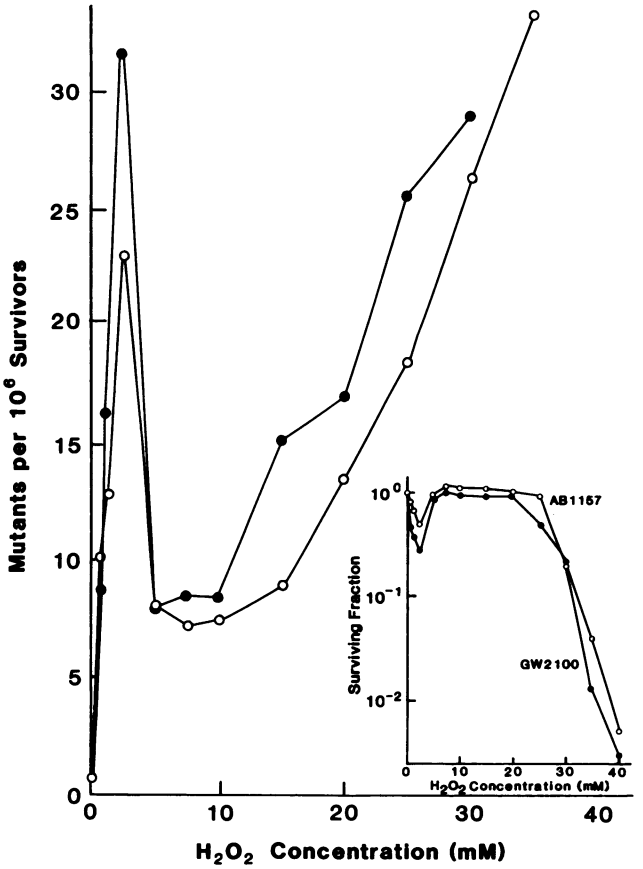

FIG. 3. Mutagenesis of *thyA* by hydrogen peroxide. Mutants were identified by growth on trimethoprim-containing plates (36). Symbols: ○, AB1157 (*umuC*$^+$); ●, GW2100 (*umuC*). (Inset) Postchallenge survival of cells on nonselective medium.

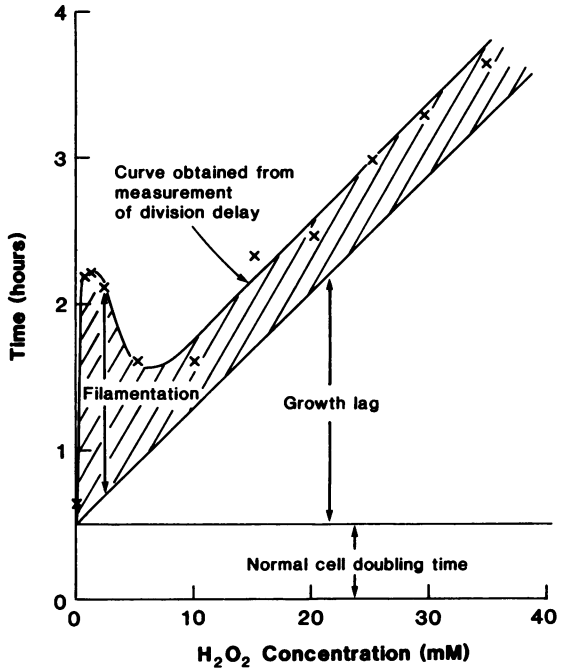

FIG. 4. Growth behavior of repair-proficient, metabolically active cells after exposure to H_2O_2. Hydrogen peroxide challenge of *E. coli* AB1157 was terminated by the addition of 2 μg of catalase, and CFU were determined at 30-min intervals. Data points (×) indicate the time elapsed after removal of H_2O_2 for a doubling of the survivor CFU. The approximate durations of the postchallenge growth lag and filamentation were determined by microscopy and are schematized in the figure. Colony-forming ability exceeded 50% at all the peroxide doses shown.

SOS induction is invoked to enhance recombinational DNA repair. Several genes whose products enhance genetic recombination are members of the SOS regulon. Foremost among these is *recA*. Under noninduced conditions, *recA* transcription is kept at levels which are sufficient to maintain basal recombination proficiency, but derepression during SOS induction causes RecA protein concentrations to increase dramatically. In *E. coli*, recombination events are thought to be mediated by either one of two pathways—one utilizes the RecBC nuclease (exonuclease V), the other utilizes *recF* function (21). Both pathways utilize RecA protein, so that the higher levels of RecA protein resulting from SOS induction might enhance the capability for recombinational DNA repair by either or both pathways.

Mutants lacking the *recBC* nuclease are sensitive to mode one killing (23). This sensitivity would be consistent with a reduction of recombinational DNA repair, but it would also occur if the nuclease was required to generate the SOS-inducing signal in peroxide-treated cells. (Such a requirement is observed in cells exposed to nalidixic acid, though not in cells treated with other SOS-inducing agents [34].) Three lines of evidence indicate that here the critical function of RecBC nuclease is for DNA recombination rather than for SOS induction. (i) When a lambda lysogen of strain JC6720 (*recB recC*) was prepared, phage induction by hydrogen peroxide occurred at a frequency just slightly lower than that of the wild-type lysogen. (The slight decrease was probably attributable to the sensitivity of the strain to killing

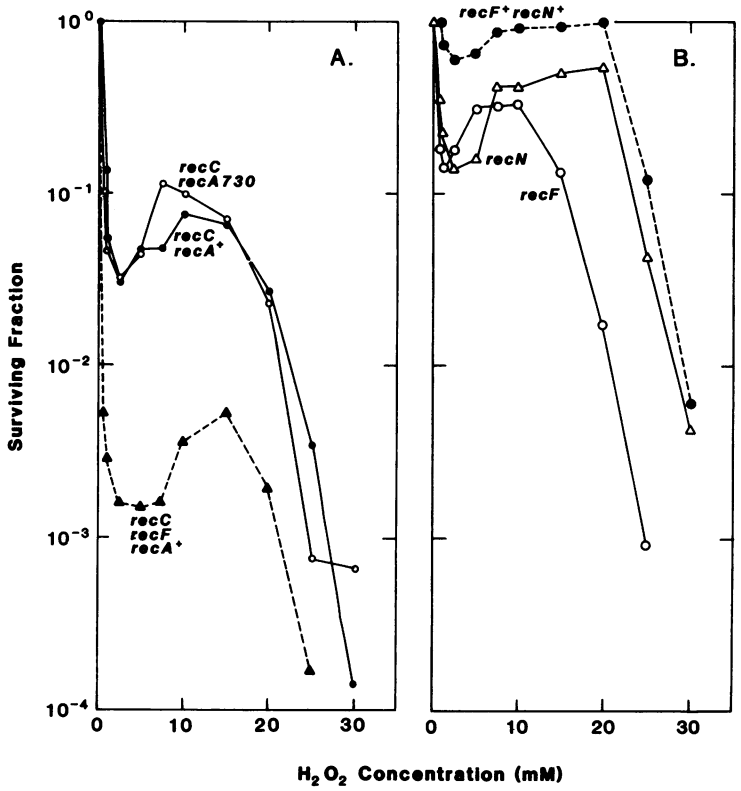

FIG. 5. Survival of *rec* mutants after challenge with H_2O_2. (A) Symbols: ●, JI120 (*recC*); ○, JI122 (*recC recA730*); ▲, JI121 (*recC recF*); ▲, JI123 (*recC recF recA730*). (B) Symbols: ●, SP648 (*recF*$^+$ *recN*$^+$); ○, JC9239 (*recF*); △, SP649 (*recN*).

by peroxide.) Therefore, RecBC nuclease must not be required to activate the RecA protease after exposure to H_2O_2. (ii) A *recC* mutation was transduced into a strain which, by virtue of a *recA730* allele, is constitutive for SOS expression (46). Although this strain exhibits enhanced UV resistance (data not shown), it was as sensitive to killing by peroxide as its *recC recA*$^+$ counterpart (Fig. 5A). (iii) The *recF* pathway can restore normal recombinational proficiency to *recBC* mutants if either of the suppressor mutations, *sbcA* or *sbcB*, are present (21). Accordingly, *recBC sbcB* (23) and *recBC sbcA* (data not shown) strains are not abnormally sensitive to mode one killing by H_2O_2.

It has been suggested that in wild-type cells, a small fraction of recombination events utilize the *recF* pathway (41). Indeed, the presence of a *recF* mutation caused a modest sensitization to mode one killing by H_2O_2, one that was considerably less than that caused by a *recBC* mutation (Fig. 5B). This sensitivity was also apparent in strains deficient in *recN*, which was identified as a member of the *recF* pathway (29) and which has been implicated in the repair of double-stranded DNA breaks (Fig. 5B). The combination of *recF* and *recC* mutations (Fig. 5A) caused a level of sensitivity to mode one killing similar to that seen in *recA* strains for which all recombinational repair is lacking (Fig. 1). Therefore, it appears that *recF*-mediated and *recBC*-mediated recombinational repair are both required for normal resistance to mode one killing and that the *recBC*-mediated pathway is normally the predominant one.

We conclude, therefore, that the enhanced protection against killing by H_2O_2 which accompanies SOS induction is mediated by an enhanced ability to perform recombinational DNA repair. The induced recombinational DNA repair is not specific to the *recF* pathway, as the latter contributes only a moderate fraction of the resistance to H_2O_2. Since the *recBC* nuclease is not SOS inducible, RecA protein is the outstanding candidate for the protective, *lexA*-regulated recombinational function. (The synthesis of single-strand binding protein is also increased after SOS induction [8], and this protein may be involved in RecA-mediated recombinational repair and in SOS induction [44]. However, the temperature-sensitive *ssb-1* allele conferred only modest sensitivity to H_2O_2 when cells were held at restrictive temperature for up to 6 h after challenge.)

Host cell reactivation of phage exposed to hydrogen peroxide. Demple and Halbrook (14) and Farr et al. (19) have observed an increase in infectivity of hydrogen peroxide-damaged phage upon infecting cells previously exposed to H_2O_2. Since the H_2O_2 doses to which the cells were exposed (60 μM) may have been sufficient to induce the SOS response, such enhanced infectivity might have been a consequence of induction of the SOS regulon. Accordingly, phage were damaged by exposure to hydrogen peroxide in the presence of copper or by exposure to UV light and then infected into wild-type cells which had been preexposed to 2.5 mM hydrogen peroxide for 15 min. This regimen stimulated reactivation of UV-damaged phage (Fig. 6A), but the infectivity of the peroxide-damaged phage was unchanged (Fig. 6B). A similar reactivation of UV-irradiated but not of H_2O_2-Cu-treated phage was observed in cells induced by UV (data not shown). SOS induction, whether by UV or by H_2O_2, apparently stimulates functions which rescue UV-irradiated but not H_2O_2-Cu-damaged phage. Evidently the reactivation of H_2O_2-treated phage observed by others was not mediated by the SOS response.

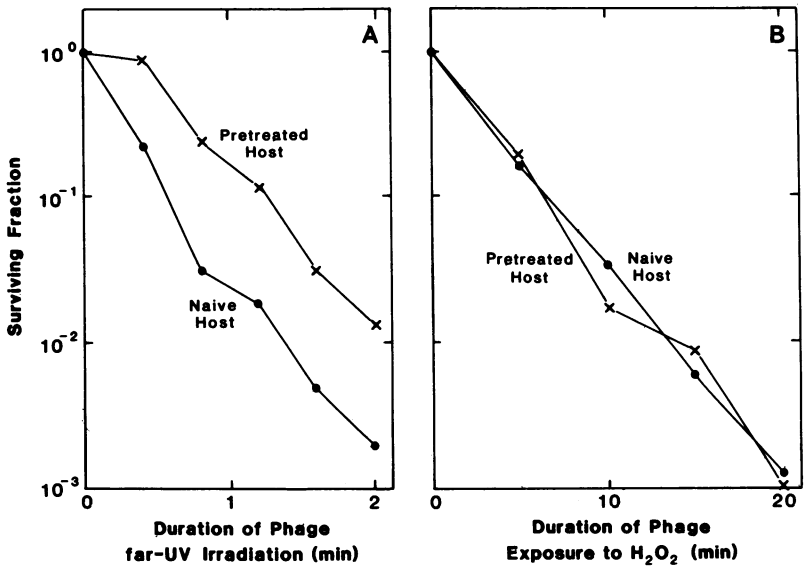

FIG. 6. Infectivity of damaged λ *vir* on naive or H_2O_2-pretreated *E. coli* AB1157. Phage and host cells were treated as described in Materials and Methods. (A) Phage preirradiated with 254-nm UV light at 1.4 J m^{-2} s^{-1}. (B) Phage preexposed to 12.5 mM H_2O_2–4 μM $CuSO_4$.

***oxyR*-directed resistance to H_2O_2 appears to be mediated via enhanced scavenging of H_2O_2.** DNA repair-proficient and -deficient strains of *E. coli* which were pretreated with low concentrations of H_2O_2 to induce the *oxyR* regulon (12, 23) exhibited a rightward shift in the dose response of killing by H_2O_2 (Fig. 7A). The magnitude of this protective effect, which is independent of the SOS response (14), increased with the density of the challenged cell population, suggesting that medium detoxification is the primary mechanism of induced resistance in these assays. Under our pretreatment regimen, catalase was induced approximately 10-fold, from 3 to 34 U/mg of protein, and pretreatment of cells which are totally deficient in catalase (*katE katG* mutants) did not result in any significant rightward shift in the dose response of killing by H_2O_2 (Fig. 7B) (27). (The concentration-independent decline in the mode one killing rate that is apparent as an upward shift in the curve in Fig. 7B was observed only in *katE katG* strains and is not understood.) Further, addition of exogenous catalase to the challenge medium of naive cells produced a rightward shift in the killing profile, and when the amount of added catalase was equal in activity to that induced in a similar number of

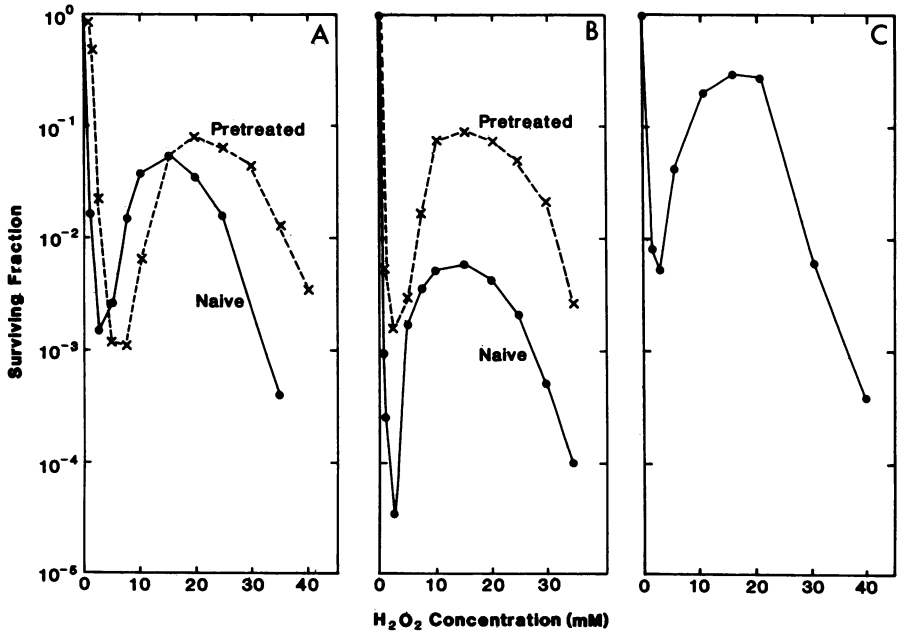

FIG. 7. Effect of scavenger enzyme deficiency on induced resistance to H_2O_2. When indicated, cells were pretreated with 60 μM H_2O_2 for 70 min before challenge. (A) JI112 (*xth katE*$^+$ *katG*$^+$). (B) JI114 (*xth katE katG*). (C) JI132 (*sodA sodB*). The superoxide dismutase-proficient parent is AB1157 (Fig. 3, inset; Fig. 8).

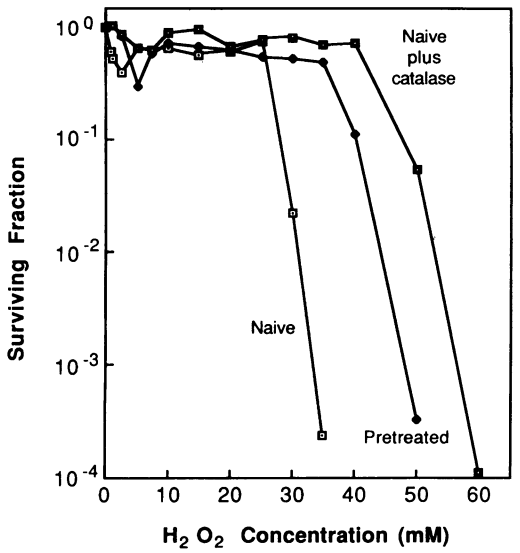

FIG. 8. Addition of exogenous catalase mimics the effect of pretreatment on subsequent resistance to H_2O_2. Naive AB1157 cells were used with no treatment (Naive). AB1157 was pretreated with 60 μM H_2O_2 for 70 min before challenge (Pretreated). An aliquot of bovine catalase (6.4 U) equivalent to the catalase induced in a similar number of pretreated AB1157 cells was added to a 10-ml naive AB1157 culture before challenge (Naive plus catalase).

induced cells, the magnitude of the shift equalled or somewhat exceeded that exhibited by the induced cells (Fig. 8).

Inducible resistance to H_2O_2 was observed in a glutathione-deficient *gshB* strain and in one that lacked both the Mn- and Fe-dependent forms of superoxide dismutase (*sodA sodB*). Moreover, total superoxide dismutase activity did not change appreciably after pretreatment of wild-type cells with H_2O_2 (data not shown). In conclusion, it appears that in these survival assays, the induction of increased levels of catalase activity is the principal effector of the *oxyR*-based inducible protection against killing by H_2O_2.

Protection by basal levels of scavenging enzymes. Loss through mutation either of catalase activity in a *katE katG* strain (27) or of glutathione in a *gshB* strain (data not shown) has no significant impact on the sensitivity of naive cells to killing by H_2O_2. *sodA* or *sodB* mutants that lack only one form of superoxide dismutase are only marginally more susceptible to mode one killing by H_2O_2 than is the sod^+ parent (data not shown). However, naive *sodA sodB* mutants that totally lack superoxide dismutase activity were sensitive to mode one killing (Fig. 7C). Moreover, the *sodA sodB* strain apparently suffers enough oxidative stress in an aerobic environment that it grows at half the rate of its parent (10). (Under anaerobic conditions the growth rates of the two strains are similar, the superoxide dismutase-deficient strain growing faster than in the presence of oxygen.) In the *sodA sodB* strain, basal levels of catalase were near normal (1 U/mg of protein), and this level increased only after exposure to peroxide. Apparently, the accumulated levels of superoxide and superoxide-mediated damage, while sufficient to significantly hinder growth of the *sodA sodB* mutant strain, do not trigger *oxyR* induction. The fact that superoxide dismutase deficiency confers sensitivity to mode one killing by H_2O_2 implies that superoxide is involved in the generation of mode one killing lesions by peroxide. In this regard, Brawn and Fridovich (9) have reported that paraquat, a bacterial agent which catalyzes the endogenous synthesis of superoxide, mediates SOS induction.

DISCUSSION

Exposure of *E. coli* to hydrogen peroxide induces at least two protective regulons: *oxyR*, which minimizes damage by an enhanced scavenging of toxic oxygen species, and SOS, which enhances recombinational DNA repair. Additional responses are also induced whose biochemical and genetic bases are unknown. A growth lag of up to 8 h was observed which may be associated with the undetermined damage that produces mode two killing, and a filamentous response was observed that was independent of the SOS response but was apparently necessary to repair lesions associated with mode one killing.

Morgan et al. (37) have noted an overlap among the many proteins induced by exposure to hydrogen peroxide and those induced by heat. Heat shock is correlated with the *htpR* regulon; however, we have observed that *htpR* mutants, which are deficient in the heat shock response, are not unusually sensitive to killing by hydrogen peroxide (23). Of course, elements of that system might still play a role in reacting to other forms of oxidative stress.

DNA recombination is an important means of repairing lesions which could cause mode one killing, and the enhancement of this ability comprises the major benefit of SOS induction. Other components of the SOS response that are vital for resisting killing by other DNA-damaging agents appear to be of little or no importance in the case of H_2O_2. Since SOS induction is thought to be a general response to DNA replication arrest, it seems reasonable than it would include functions whose deficiency could be aphenotypic for some SOS-inducing treatments.

That the sensitivities conferred by the absence of the *recBC* and *recF* pathways of genetic recombination are approximately additive suggests that each pathway normally has an independent class of damage as its substrate. We do not know precisely which lesions these pathways correct, though Picksley et al. (41) have speculated that RecN protein (thought to be a member of the *recF* pathway) is required to repair double-strand breaks. However, the *recF* pathway can function effectively in lieu of the *recBC* pathway in the presence of *sbcB* or *sbcA* mutations (23), suggesting that a strict division of substrate specificity for the two pathways does not exist.

While the SOS response is apparently invoked to alleviate mode one killing by enhancing DNA recombinational repair, other mechanisms of DNA repair also contribute to survival. Exonuclease III and DNA polymerase I mutants are both extremely sensitive to mode one killing, and since loss of either function confers additional sensitivity on recombination-deficient strains (23, 38), each must play a role in nonrecombinational excision DNA repair.

That peroxide-induced mutagenesis appears to be independent of *umuC* is somewhat at odds with the report (26) that peroxide-directed mutagenesis in *Salmonella typhimurium* is enhanced by the presence of pKM101, a plasmid that encodes the MucC and MucD proteins which are considered to be analogous to the UmuC and UmuD proteins in *E. coli*. However, the trimethoprim selection used in this *E. coli* study allows detection of many mutational events, e.g., inversions, insertions, deletions, which would not be seen in the specific base-substitution reversion assay used in the *S. typhimurium* study. Perhaps MucC and MucD proteins are requisite only for the latter type of event.

The dose response of mode one killing by hydrogen peroxide is a maximum rate of killing at 1 to 3 mM and a half-maximal dose-independent rate at higher concentrations. Previous work has suggested that this pattern reflects rates of DNA damage production, not those of DNA repair (23). In support of that conclusion, we report here that phage lambda induction, mutagenesis, filamentation, and UV-irradiated phage reactivation proficiency induced by H_2O_2 also exhibit submaxima at 1 to 3 mM peroxide. We have speculated on a metabolic pathway that might activate peroxide and have proposed possible chemical and biochemical explanations that could produce the peculiar mode one killing dose response (27). However, the specific DNA lesions responsible for mode one killing and the precise mechanism of their formation remain to be identified.

The lesions responsible for mode two killing are also unknown, but the facts that mutagenesis increases with exposure to H_2O_2 in the dose range which yields mode two killing (Fig. 3) and that *polA* mutants appear to be extremely sensitive to mode two killing (23) imply that DNA damage is involved. Why mode two killing lesions appear to give a growth delay and whether non-DNA targets are also involved remain to be elucidated.

While the *oxyR*-directed synthesis of catalase results in a marked shift in mode two killing to higher concentrations of exogenous H_2O_2, the lessening of mode one killing by low concentrations of peroxide might have greater physiological significance. Pretreatment lessens the rate of mode one killing at 0.75 mM H_2O_2 by about a factor of 20 in a repair-deficient strain (Fig. 7A), implying the existence of an accordant decrease in the amount of DNA damage.

Hydrogen peroxide may be a periodic threat to aerobic organisms, since aerobic metabolism produces hydrogen peroxide as a by-product of biosynthesis and respiration (6, 16). Indeed, DNA damage can accumulate to toxic levels in some DNA-repair-defective mutants of *E. coli*, so that these strains can be easily maintained only when anaerobically grown (23). Bacteria appear to have evolved multiple mechanisms by which to protect themselves against their oxidative environment, but whether some or all of these strategies also are used by eucaryotic organisms is unknown. This point may be particularly significant in higher organisms that use oxygen radical generation as a mechanism to defeat invasive cells (20) or in which injured tissue may generate H_2O_2 after interruption and resumption of oxygen availability (32). Moreover, results from studies of H_2O_2 are directly applicable to chemicals or radiation which mediate cell damage via the generation of either H_2O_2 or free radical species which are produced by H_2O_2.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM19020 and P30ES01896 from the National Institutes of Health and by a National Science Foundation graduate fellowship to J.A.I.

We are greatly appreciative of the generosity of our colleagues who donated bacterial strains as noted in Table 1.

LITERATURE CITED

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol Rev. **36**:525–557.
2. **Bagg, A., C. J. Kenyon, and G. C. Walker.** 1981. Inducibility of a gene product required for UV and chemical mutagenesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **78**:5749–5753.
3. **Barbour, S. D., and A. J. Clark.** 1970. Biochemical and genetic studies of recombination proficiency in *Escherichia coli*. I. Enzymatic activity associated with *recB*$^+$ and *recC*$^+$ genes. Proc. Natl. Acad. Sci. USA **65**:955–961.
4. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. **44**:276–287.
5. **Beers, R. F., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195**:133–140.
6. **Boveris, A., and E. Cadenas.** 1982. Production of superoxide radicals and hydrogen peroxide in mitochondria, p. 15–30. *In* L. W. Oberly (ed.), Superoxide dismutase. CRC Press, Inc., Boca Raton, Fla.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
8. **Brandsma, J. A., D. Bosch, C. Backendorf, and P. van de Putte.** 1983. A common regulatory region shared by divergently transcribed genes of the *Escherichia coli* SOS system. Nature (London) **305**:243–245.
9. **Brawn, M. K., and I. Fridovich.** 1985. Increased superoxide radical production evokes inducible DNA repair in *Escherichia coli*. J. Bacteriol. **260**:922–925.
10. **Carlioz, A., and D. Touati.** 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? EMBO J. **5**:623–630.
11. **Casadaban, M. J., and S. N. Cohen.** 1980. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76**:4530–4533.
12. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41**:752–762.
13. **Clark, A. J., and A. D. Margulies.** 1965. Isolation and characterization of recombination-deficient mutants of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA **53**:451–459.
14. **Demple, B., and J. Halbrook.** 1983. Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature (London) **304**:466–468.
15. **Devoret, R., M. Pierre, and P. L. Moreau.** 1983. Prophage θ80 is induced in *Escherichia coli* K12 *recA430*. Mol. Gen. Genet. **189**:199–206.
16. **Dixon, M., and E. C. Webb.** 1979. Enzymes, 3rd ed. Academic Press, Inc., New York.
17. **Elledge, S. J., and G. C. Walker.** 1985. Proteins required for ultraviolet light and chemical mutagenesis. Identification of the products of the *umuC* locus of *Escherichia coli*. J. Mol. Biol. **164**:175–192.
18. **Farr, S. B., R. D'Ari, and D. Touati.** 1986. Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. Proc. Natl. Acad. Sci. USA **83**:8268–8272.
19. **Farr, S. B., D. O. Natvig, and T. Kogoma.** 1985. Toxicity and mutagenicity of plumbagin and the induction of a possible new DNA repair pathway in *Escherichia coli*. J. Bacteriol. **164**: 1309–1316.
20. **Gabig, T. K., and B. M. Babior.** 1982. Oxygen-dependent microbial killing by neutrophils, p. 1–14. *In* L. W. Oberly (ed.), Superoxide dismutase. CRC Press, Inc., Boca Raton, Fla.
21. **Horii, Z., and A. J. Clark.** 1973. Genetic analysis of the recF pathway to genetic recombination in *Escherichia coli* K12: isolation and characterization of mutants. J. Mol. Biol. **80**: 327–344.
22. **Huisman, O., and R. D'Ari.** 1981. An inducible DNA replication-cell division coupling mechanism in *E. coli*. Nature (London) **290**:797–799.
23. **Imlay, J. A., and S. Linn.** 1986. Bimodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. J. Bacteriol. **166**:519–527.
24. **Kenyon, C. J., and G. C. Walker.** 1980. DNA-damaging agents stimulate gene expression at specific loci in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77**:2819–2823.
25. **Kumura, K., M. Sekiguchi, A.-L. Steinum, and E. Seeberg.** 1985. Stimulation of the UvrABC enzyme-catalyzed repair reactions by the UvrD protein (DNA helicase II). Nucleic Acids Res. **13**:1483–1492.

26. **Levin, D. E., M. Hollstein, M. F. Christman, E. A. Schwiers, and B. N. Ames.** 1982. A new *Salmonella* tester strain (TA102) with A:T base pairs at the site of mutation detects oxidative mutagenesis. Proc. Natl. Acad. Sci. USA **79**:7445–7449.
27. **Linn, S., and J. A. Imlay.** 1987. Toxicity, mutagenesis, and stress responses induced in *Escherichia coli* by hydrogen peroxide. J. Cell Sci. **6**(Suppl.):289–301.
28. **Little, J. W., S. H. Edmiston, L. Z. Pacelli, and D. W. Mount.** 1980. Cleavage of the *Escherichia coli lexA* protein by the *recA* protease. Proc. Natl. Acad. Sci. USA **77**:3225–3229.
29. **Lloyd, R. G., S. M. Picksley, and C. Prescott.** 1983. Inducible expression of a gene specific to the RecF pathway for recombination in *Escherichia coli* K12. Mol. Gen. Genet. **190**:162–167.
30. **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.
31. **Markham, B. E., J. W. Little, and D. W. Mount.** 1981. Nucleotide sequence of the *lexA* gene of *Escherichia coli* K12. Nucleic Acids Res. **9**:4149–4161.
32. **McCord, J. M.** 1985. Oxygen-derived free radicals in postischemic tissue injury. N. Eng. J. Med. **312**:159–163.
33. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase, an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. **244**:6049–6055.
34. **McPartland, A., L. Green, and H. Echols.** 1980. Control of *recA* gene RNA in *E. coli*: regulatory and signal genes. Cell **20**: 731–737.
35. **Milcarek, C., and B. Weiss.** 1972. Mutants of *Escherichia coli* with altered deoxyribonucleases. Isolation and characterization of mutants for exonuclease III. J. Mol. Biol. **68**:303–318.
36. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz, and B. N. Ames.** 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc. Natl. Acad. Sci. USA **83**:8059–8063.
38. **Morimyo, M.** 1982. Anaerobic incubation enhances the colony formation of a *polA recB* strain of *Escherichia coli* K-12. J. Bacteriol. **152**:208–214.
39. **Mount, D. W., K. B. Low, and S. J. Edmiston.** 1972. Dominant mutations (*lex*) in *Escherichia coli* K12 which affect radiation sensitivity and frequency of ultraviolet light-induced mutations. J. Bacteriol. **112**:886–893.
40. **Pang, P. P., and G. C. Walker.** 1983. Identification of the *uvrD* gene product of *Salmonella typhimurium* LT2. J. Bacteriol. **153**:1172–1179.
41. **Picksley, S. M., P. V. Attfield, and R. G. Lloyd.** 1984. Repair of DNA double-strand breaks in *Escherichia coli* K12 requires a functional *recN* product. Mol. Gen. Genet. **195**:267–274.
42. **Roberts, J. W., and C. W. Roberts.** 1981. Two mutations that alter the regulatory activity of *E. coli* recA protein. Nature (London) **290**:422–424.
43. **Roberts, J. W., C. W. Roberts, and N. L. Craig.** 1978. *Escherichia coli recA* gene product inactivates phage λ repressor. Proc. Natl. Acad. Sci. USA **75**:4714–4718.
44. **Vales, L. D., J. W. Chase, and J. B. Murphy.** 1980. Effect of *ssbA1* and *lexC113* mutations on lambda prophage induction, bacteriophage growth, and cell survival. J. Bacteriol. **143**:887–896.
45. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol. Rev. **48**:60–93.
46. **Witkin, E. M., J. O. McCall, M. R. Volkert, and I. E. Wermundsen.** 1982. Constitutive expression of SOS functions and modulation of mutagenesis resulting from resolution of genetic instability at or near the *recA* locus of *Escherichia coli*. Mol. Gen. Genet. **185**:43–50.