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Genetics

Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase

(oxygen radicals/DNA lesions/exonuclease III)

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ABSTRACT **_Escherichia coli_ double mutants (*sodA sodB*) completely lacking superoxide dismutase (SOD) have greatly enhanced mutation rates during aerobic growth. Single mutants lacking manganese SOD (MnSOD) but possessing iron SOD (FeSOD) have a smaller increase, and single mutants lacking FeSOD but possessing MnSOD do not show such an increase. The enhancement of mutagenesis is completely dependent on the presence of oxygen, and treatments that increase the flux of superoxide radicals produce even higher levels of mutagenesis. The presence of a plasmid overproducing either form of SOD reduces the level of mutagenesis to that of wild type, showing that the O_2-dependent enhancement results from a lack of SOD. The enhancement of mutagenesis is RecA-independent, and a complete lack of SOD does not induce the SOS response during aerobic growth. However, the enhanced mutagenesis in aerobically grown *sodA sodB* mutants is largely dependent on functional exonuclease III, suggesting that the increased flux of superoxide radicals results in DNA lesions that can be acted on by this enzyme, leading to mutations.**

Within aerobically growing cells, molecular dioxygen is reduced by a number of pathways to produce the superoxide radical, $O_2^{-\cdot}$ (1, 2). The reactivity of O_2 radicals has been questioned (3, 4), although there is increasing evidence that $O_2^{-\cdot}$ can directly damage macromolecules (5, 6). In addition, $O_2^{-\cdot}$ readily undergoes further reduction to form H_2O_2 and $OH^{\cdot}$ radicals, both of which are damaging to macromolecules, including DNA (7, 8). Further evidence suggesting that $O_2^{-\cdot}$ is harmful to cells is the fact that the vast majority of organisms studied to date contain the enzyme superoxide dismutase (SOD), which catalyzes the dismutation of O_2 radicals (1). The bacterium *Escherichia coli* contains two forms of SOD, manganese SOD (MnSOD) encoded by the *sodA* gene, and iron SOD (FeSOD) encoded by the *sodB* gene (9, 10). *sodB* is constitutive and *sodA* is inducible under increased levels of O_2 (11, 12). Hyperbaric oxygen has been shown to be mutagenic and toxic to wild-type *E. coli*, although there is little direct evidence that superoxide radicals are responsible (13, 14).

To increase the flux of O_2 radicals within the cell, investigators have used compounds such as paraquat or plumbagin, which produce O_2 radicals by redox cycling (15). These compounds are not totally satisfactory in that they probably produce reactive species other than O_2 radicals (16). In addition, although both plumbagin and paraquat strongly induce MnSOD activity, their effects on the cell are somewhat distinct. For example, paraquat and plumbagin divert electrons from different sources within the cell (15). Furthermore, in *Salmonella typhimurium* paraquat and naphthoquinones similar to plumbagin produce different polyphosphorylated dinucleotides, which have been suggested to be "alarmones" (17, 18).

Reports vary on the mutagenicity of plumbagin and paraquat (19–21). Such mutagenicity has been suggested to arise from the increased flux of O_2 radicals these compounds produce in the presence of oxygen. However, one cannot discount the possibility that the observed mutagenesis derives from other reactants produced by such compounds or from the induction of an error-prone repair system. Indeed, it has recently been reported that paraquat induces the SOS response in *E. coli* (22), which is mutagenic to both damaged and undamaged DNA (23). Another problem associated with the use of redox cycling compounds such as plumbagin and paraquat is that they are highly toxic at the concentrations required to observe significant mutagenesis (refs. 19 and 21; unpublished observations).

Because superoxide radicals are produced as a by-product of normal aerobic metabolism, a more direct approach to ascertaining their mutagenic potential would be to assay oxygen-dependent mutagenesis in cells lacking SOD. We recently reported the construction of *E. coli* double-mutant strains lacking both FeSOD and MnSOD. These mutants grow aerobically at about one-half the rate of the wild type in rich medium, but they are unable to grow on minimal medium (24). In this paper, we present evidence that mutation rates are greatly increased in the absence of functional SOD and that this increase is entirely dependent on the presence of molecular oxygen.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. Bacterial strains and plasmids are listed in Table 1. Precultures were generally grown in LB broth (26) at 37°C with appropriate antibiotics. Experimental cultures did not contain antibiotics. Anaerobic cultures were supplemented with 1% glucose. Minimal salts medium was M63 (26). P1 transductions and transformation with plasmids were performed as described elsewhere (26, 27). Antibiotics were used at the following concentrations: chloramphenicol, 10 μg/ml; kanamycin, 40 μg/ml; tetracycline, 12.5 μg/ml; and ampicillin, 50 μg/ml.

Growth in Plumbagin and Paraquat. Precultures were grown in LB broth with appropriate antibiotics to an OD_{600} of 1.0 and chilled on ice. These were diluted 1:100 in LB broth and shaken at 145 rpm at 37°C; samples were taken at indicated times for optical density measurement. Plumbagin or paraquat was added during exponential growth (OD_{600}, 0.2).

Use of the *sfiA::lac* Fusion to Monitor SOS Induction. Cells were lysogenized with λp(*sfiA::lac*)c*Ind* as described (28). β-Galactosidase activity was measured according to Miller

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Abbreviation: SOD, superoxide dismutase.

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Table 1. *E. coli* strains and plasmids

	Genotype	Source or ref.
Strain		
GC4468	F⁻Δlac U169 *rpsL*	R.D.
QC772	as GC4468 butϕ(*sodA–lacZ*)49 Cm^R	24
QC773	as GC4468 butϕ(*sodB–kan*)1–Δ2 Km^R	24
QC774	as GG4468 butϕ(*sodA–lacZ*)49 Cm^R (*sodB–kan*)1–Δ2 Km^R	24
QC905	as QC774 but *recA56 srlC300*::Tn*10*	P1.JC10240 × QC774 selectTc^R
BW35	*thi-1 relA1 spoT1*	B. Weiss
QC909	as QC773 but (*sodA*::MudPR13)25 Cm^R	24
QC910	as BW35 but *sodA sodB*	P1.QC772 × BW35 select Cm^R then P1.QC773 select Km^R
JC10240	Hfr PO45 *srlC300*::Tn*10 recA56 thr ilv rpsE*	25
BW295	*thi-1 relA1 spoT1 xthA*	B. Weiss
QC915	as BW295 but *sodA sodB*	P1.QC772 × BW295 select Cm^R then P1.QC773 select Km^R
GC694	F⁻*recA441*(Tif)*sfiB103 hisG4 thr pro arg rpsL lac gal*	R.D.
QC973	as GC4468 but *recA56 srlC300*::Tn*10*	P1.JC10240 × GC4468 select Tc^R
Plasmid		
pDT1.5	$sodA^+$ Ap^R	9
pHS1.8	$sodB^+$ Tc^R	10
pSGR1	xth^+ Ap^R Km^R	B. Demple

All strains are K-12 derivatives. Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance; Tc^R, tetracyclin resistance; Ap^R, ampicillin resistance.

(26), and the differential rate of synthesis was calculated as described (29).

RESULTS

Spontaneous Mutagenesis in sod Mutants. We used two assays to measure mutagenesis in various sod mutants. The rate of mutagenesis was determined by a fluctuation test measuring the rate of rifampicin-sensitive to rifampicin-resistant mutagenesis. Mutagenesis was also monitored by assaying the frequency of thymine-negative (Thy^-) mutants.

The target of the antibiotic rifampicin is the β subunit of the RNA polymerase, and resistant mutants possess an altered β subunit due to mutations in the *rpoB* gene (30). Such mutations must be mostly. if not exclusively, base substitutions. The mutation rate toward rifampicin resistance is thus a sensitive assay for this class of mutational events. We measured these rates using modified fluctuation tests. The sodA sodB double mutant showed a 40-fold increase in the rate of spontaneous mutation toward rifampicin resistance, and the sodA single mutant showed a 9-fold increase compared to the wild type; the sodB single mutant did not show an increase in the mutation rate (Table 2). Introduction in the double mutant of a multicopy plasmid carrying a functional *sodA* gene (pDT1.5) reduced the mutation rate to that of the wild type. It is thus the lack of SOD that is responsible for the dramatic increase in mutation rate.

Thy^- mutants are resistant to the drug trimethoprim and can be selected from a Thy^+ population (26). We used this simple selection to measure the frequency of thyA mutants in wild-type and mutant cultures. The sodA sodB double mutant culture had a significantly higher mutant frequency than the wild type (Table 3). The sodA culture also had a slightly increased mutant frequency; the sodB culture did not. The presence of a multicopy plasmid carrying $sodA^+$ or $sodB^+$ in the sodA sodB mutant strain reduced the mutant frequency to the level of the wild type (Table 3), showing that the increase was due to lack of SOD activity. For all strains used, at least 10 trimethoprim-resistant clones were tested, and all were found to be Thy^-, so the frequencies measured were those of Thy^- mutants.

We also attempted to measure mutagenesis by determining the reversion frequency in hisG4 auxotrophic strains. Again, the sodA sodB strain had the highest frequency of His^+ revertants. The sodA strain also had an increased frequency of revertants, whereas sodB had the same frequency as the wild type (data not shown). However, auxotrophic reversion tests required plating cells on supplemented minimal medium (minimal salts, glucose, B1, 19 amino acids plus limiting histidine), and sodA sodB double mutants grow poorly on this minimal medium (24), complicating the interpretation of the results.

Increased Mutagenesis in sod Mutants Depends on $O_2^{\cdot-}$ Flux. In anaerobic cultures, the frequency of Thy^- mutants was essentially identical for the sodA sodB double-mutant and the wild-type strain (Table 3). This mutant frequency was only slightly lower than that found in wild-type aerobic cultures.

Increased partial pressure of oxygen and plumbagin cause an increase in the intracellular flux of O_2 radicals. When

Table 2. $Rif^S \rightarrow Rif^R$ mutation rate

Strain	Relevant genotype	Mutation rate	Relative mutation rate
GC4468	Wild type	6.6×10^{-10}	1.0
QC773	*sodB*	6.4×10^{-10}	0.9
QC772	*sodA*	5.7×10^{-9}	8.7
QC774	*sodA sodB*	2.7×10^{-8}	41.0
QC774/pDT1.5	*sodA sodB*/$sodA^+$	7.2×10^{-10}	1.1

The mutation rate a is the number of mutations per cell per generation and is given by the formula $a = (-\ln P_0)/N$, where P_0 is the ratio of clear tubes to tubes containing rifampicin-resistant (Rif^R) mutants and N is the actual number of cells in each tube at the time of rifampicin addition. Values given are the average of at least two independent experiments. Variation did not exceed 30%. Rifampicin sensitive (Rif^S) cells were grown to $OD_{600} = 0.1$ in LB broth and then diluted to <500 cells per ml in LB broth. A sample was plated to determine the actual titer. Then, 0.5 ml was placed into each of 24 tubes and incubated at 37°C; the cultures were shaken at 150 rpm until they reached an OD_{600} of ≈0.1 for *sodA sodB* double mutants and 1.0 for wild-type cells. (Because of the difference in mutation rates, rifampicin was added to the double mutants at a lower cell density than for the wild type.) Samples were again plated to determine the actual titer and then 0.5 ml of LB broth containing rifampicin at 160 μg/ml was added to each tube. Incubation was continued as before for an additional 12–24 hr. All saturated tubes were restreaked to check that they were not contaminants; these cultures were assumed to have contained at least one Rif^R mutant at the time of rifampicin addition. Calculation of the mutation rate was as described by Luria and Delbrück (31).

Table 3. $Thy^+ \rightarrow Thy^-$ forward mutation

Strain	Genotype	Thy^- mutants per 10^7 cells* plated on LBT plus trimethoprim			
		Aerobic	Oxygenated	Plumbagin†	Anaerobic
GC4468	Wild type	21	18	32	15
QC773	*sodB*	14	ND	21	ND
QC772	*sodA*	25	ND	243	ND
QC774	*sodA sodB*	121	1520	513	13
QC774/pDT1.5	*sodA sodB*/*sodA*$^+$	19	16	25	ND
QC778/pHS1.8	*sodA sodB*/*sodB*$^+$	21	ND	ND	ND

Thy^+ cells were grown in LB broth at 37°C aerobically, hyperoxygenated, or anaerobically to an OD_{600} of ≈1.0. Samples were plated directly on LB plates containing thymine at 50 μg/ml and trimethoprim at 15 μg/ml and diluted on LB plates without trimethoprim to determine the titer. Hyperoxygenation was achieved by bubbling pure oxygen through 5-ml cultures with a Pasteur pipette. For anaerobic experiments, precultures were grown anaerobically for 48 hr before beginning the experiment. Values given are the average of two plates (colony counts ranged from 39 to 602 per plate) and each experiment was repeated at least twice without significant variation. *sodA sodB* thy^- cells did not show any growth advantage over thy^+ cells as tested by a thy^-/thy^+ mixed culture experiment where cells were grown 12 generations without a measurable change from the initial ratio of thy^-/thy^+. The efficiency of plating was ≥90% in all strains and conditions (save for plumbagin) and was determined by comparison with particle count using a Coulter cell counter. The efficiency of plating for plumbagin-treated cells ranged from 50% to 70% for all strains save the *sodA sodB* double mutant, which gave 30%. ND, not determined.
*As determined by viable counts.
†Plumbagin concentration was 0.5 mM.

cultures of a sodA sodB double mutant were exposed to hyperbaric oxygen or plumbagin, they displayed a very high frequency of Thy^- mutants compared to the wild-type strain, which showed only a slight increase (Table 3). The *sodA* plasmid (pDT1.5) reduced the mutant frequency of the sodA sodB strain to wild-type levels. The above results strongly suggest that the increase in spontaneous mutagenesis in cells lacking SOD depends on the presence of oxygen, and that exposure to increased levels of O_2 radicals greatly enhances mutagenesis in these mutants.

Oxidative Mutagenesis in sod Mutants Is SOS Independent. Many conditions that lead to DNA damage or an inhibition of DNA synthesis activate the protease activity of the RecA protein, resulting in cleavage of the LexA repressor and induction of the SOS response, which includes mutagenic repair (23, 32, 33). This induction is abolished in recA mutants. The presence of a *recA* mutation did not alter the oxygen-dependent enhancement of mutants in sodA sodB cultures, however (Table 4). Thus, the observed mutagenesis of sod mutants exposed to oxygen is independent of the SOS response.

Although the enhanced mutagenesis in sodA sodB strains does not require induction of the SOS response, it nevertheless remained possible that this response is in fact induced by the increased $O_2^{-\cdot}$ flux. To determine whether the SOS response was induced in sodA sodB mutants during aerobic growth, we used a *sfiA*::*lacZ* operon fusion. The *sfiA*::*lac* fusion makes β-galactosidase an artificial SOS function and thus a direct indicator of the induction of the SOS response (34). Using a sodA25 sodB (λ*sfiA*::*lac cIind*) strain, we found that the SOS response was not induced during aerobic growth, although it remained inducible by naladixic acid: the differential rate of β-galactosidase synthesis was 120 units/mg (dry wt), increasing to 2500 units/mg (dry wt) in the presence of naladixic acid at 40 μg/ml.

Table 4. Mutagenesis in *recA* strains

Strain	Genotype	$Thy^+ \rightarrow Thy^-$, mutants per 10^7 viable cells	$Rif^S \rightarrow Rif^R$, mutations per cell, per generation
QC973	*recA*	14	1.4×10^{-9}
QC774	*sodA sodB*	87	2.8×10^{-8}
QC905	*sodA sodB recA*	99	2.7×10^{-8}

The SOS response includes error-prone repair functions that create mutations at the site of DNA lesions—"targeted" mutagenesis—and functions that mutate undamaged DNA—"untargeted" mutagenesis (35). It is possible, however, that all DNA bears cryptic lesions, revealed only when fixed as mutations in an SOS-induced cell. Active oxygen species are a potential source of such lesions. We therefore investigated the possibility that untargeted SOS mutagenesis is oxygen dependent, measuring His^+ reversion in a *recA*(Tif) *ftsZ*(SfiB) *hisG4* strain growing aerobically or anaerobically. The *recA*(Tif) mutation induces the SOS response spontaneously at 42°C (23), and the *ftsZ*(SfiB) mutation suppresses SOS-associated division inhibition (36). The results (Table 5) reveal no reduction in Tif-mediated untargeted mutagenesis in the absence of oxygen. We conclude that SOS untargeted mutagenesis is not responding to oxygen-dependent lesions.

Role of Exonuclease III on Mutagenesis and Growth in sod Mutants. Exonuclease III, encoded by the *xth* gene, has several catalytic activities that are involved in the repair of certain types of DNA damage. The enzyme possesses 3′ exonuclease and phosphatase activities, RNase H, and apurinic endonucleolytic activities, as well as an endonucleolytic activity that recognizes bases containing urea residues (37–40). xth mutants lack 90% of the normal exonuclease III

Table 5. Tif-mediated mutagenesis with and without oxygen

Aerobic			Anaerobic		
30°C	42°C	42°C/30°C	30°C	42°C	42°C/30°C
10	292		5	167	
14	292		9	199	
18	287		11	223	
	Average			Average	
14	290	21	8	196	24

Numbers listed indicate the number of His^+ revertants per plate. Strain GC694 was grown aerobically at 30°C in glucose Casamino acids medium to ≈10^8 cells/ml, washed, and plated (10^7 cells per plate) on minimal glucose (0.4%) medium supplemented with threonine, proline, arginine (100 μg/ml each), thiamine (10 μg/ml), adenine (100 μg/ml), and limiting histidine (0.8 μg/ml). Plates were incubated aerobically and anaerobically at 30°C and 42°C.

activities (39) and are highly sensitive to certain types of oxidative stress (8, 41, 42). We were therefore interested in the effect of an *xth* mutation on growth and mutagenesis in sod mutants. As previously reported, sodA sodB double mutants grow aerobically in rich media (24). Introduction of sodA sodB into an xth background did not affect the aerobic growth rate (Fig. 1*A*). The sensitivity to paraquat and plumbagin, however, already considerable in sodA sodB double mutants, increased even further in the sodA sodB xth triple mutant (Fig. 1*B*). Thus, the presence of functional exonuclease III diminishes the effects of O_2 radical-dependent damage in cells lacking SOD, implying that this damage includes DNA lesions.

The hypersensitivity of the sodA sodB xth triple mutant to plumbagin and paraquat suggests that exonuclease III may recognize DNA lesions produced under conditions of increased $O_2^{\cdot-}$ levels. When we measured mutant frequencies in wild-type, sodA sodB, and sodA sodB xth cultures, we found that the enhanced frequency observed in the sodA sodB culture was largely dependent on the presence of a functional *xth* gene (Table 6). Enhanced $O_2^{\cdot-}$ mutagenesis was again observed when a plasmid containing a functional xth gene (pSGR1) was introduced into the sodA sodB xth triple mutant (Table 6). These observations suggest that cells lacking SOD suffer from increased levels of premutagenic DNA damage, and that the action of exonuclease III on these DNA lesions greatly enhances their mutagenicity.

Table 6. $Thy^+ \rightarrow Thy^-$ in sod xth strains

Strain	Genotype	Mutants per 10^7 cells
BW35	Wild type	23
BW295	*xth*	10
QC910	*sodA sodB*	120
QC915	*sodA sodB xth*	33
QC915/pSGR1	*sodA sodB xth/xth*$^+$	280

DISCUSSION

Normal metabolism in the presence of oxygen produces superoxide radicals (43). Evidence for the direct or indirect toxicity of these radicals has accumulated (13, 14, 19, 44). Most organisms that live in aerobic environments possess enzymatic superoxide radical scavengers, SODs which catalyze the dismutation of these radicals, suggesting that it may be essential to protect cells against O_2 radicals or their derivatives (1). However, although numerous investigations have supported this hypothesis (reviewed by Fridovich in ref. 1), the physiological role of SOD remained speculative until recently. The isolation of *E. coli* mutants completely devoid of SOD permitted the demonstration that SOD efficiently protects cells against oxidative damage caused by oxygen, H_2O_2, and paraquat; furthermore, no growth of these mutants was observed in aerobic conditions requiring amino acid biosynthesis by the cell (24). Also, it has recently been reported that mutations in the cytoplasmic SOD-encoding allele *csod* of *Drosophila melanogaster* are lethal (45).

In the present work, we have demonstrated that *E. coli* mutants devoid of SOD, although able to grow aerobically in rich media, are subject to an increased level of oxygen-dependent mutagenesis compared to the background level in sod^+ cells. Mutagenesis in strains lacking SOD is increased even further under conditions that increase the intracellular flux of O_2 radicals, and the enhanced mutagenesis in these mutants disappears when cells are grown anaerobically. We conclude that O_2 radicals can lead to mutations and that one physiological role of SOD is to prevent this mutagenesis.

Most organisms possess two forms of SOD. In eukaryotic organisms, these are localized differently (CuSOD in the cytoplasm and MnSOD in the mitochondria); in prokaryotic cells compartmentalization is not likely. In general, one *sod* gene is constitutive, and the other is inducible by O_2 (1). In the facultative aerobic bacterium *E. coli*, the *sodB* gene encoding FeSOD is expressed constitutively, whereas *sodA*, encoding MnSOD, is inducible. We found no increased mutagenesis in sodB mutants, whereas there was an increase in sodA mutants, although in normal aerobic conditions, MnSOD and FeSOD levels are about the same in wild-type cells (refs. 15 and 24; unpublished results). However, a different role for the two enzymes in regard to protection against mutagenesis cannot explain this observation since (*i*) mutagenesis in the double mutant is greatly increased compared to the sodA single mutant, showing that both enzymes are protective, and (*ii*) an excess of either MnSOD or FeSOD, provided by a multicopy plasmid, protects the double mutant equally well. It is therefore likely that the better protection provided by MnSOD in sodB mutants is directly related to its inducibility.

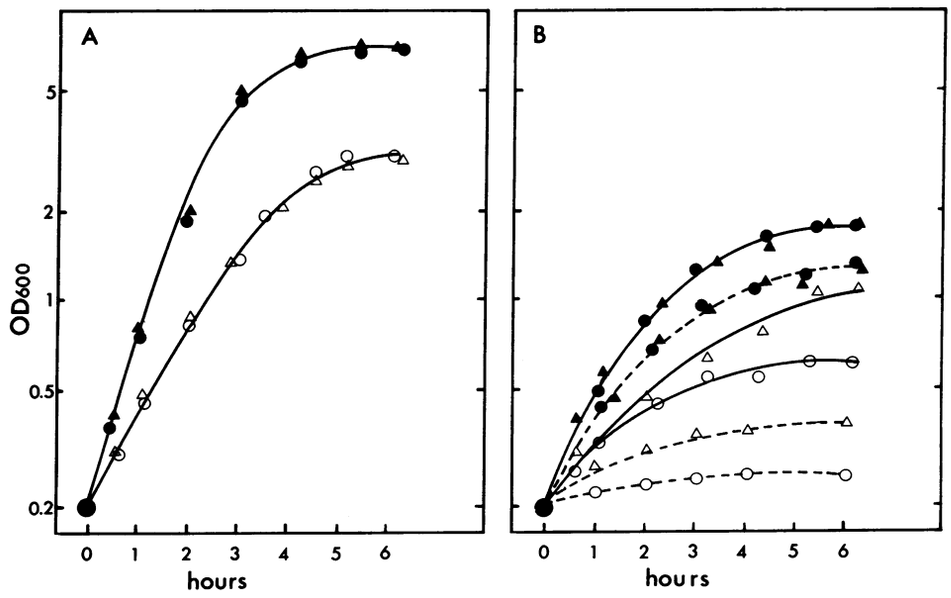

FIG. 1. Aerobic growth of wild-type (BW35, ●), xth (BW295, ▲), sodA sodB (QC910, △), and sodA sodB xth (QC915, ○) in LB broth at 37°C with no additives (*A*) or in the presence of 75 μM paraquat (*B*, solid lines) or 500 μM plumbagin (*B*, broken lines), added at time zero.

The oxygen-dependent enhancement of mutagenesis observed in sodA sodB double mutants was RecA independent. Thus, the SOS response is not responsible for this mutagenesis. We recently reported evidence suggesting the existence of an SOS-independent DNA repair response induced by an increase in the flux of intracellular O_2 radicals (21). We need to determine whether this induced repair is error prone.

Exonuclease III, the product of the *xth* gene, has several functions involved in DNA repair, and it has been demonstrated to recognize DNA lesions caused by oxidizing agents (38, 40). We compared the effects of plumbagin and paraquat on sodA sodB double mutants and sodA sodB xth triple mutants. The presence of functional exonuclease III enhanced the growth of a sodA sodB mutant exposed to an increased flux of O_2 radicals, and it was also required for the full enhancement of mutagenesis. These observations strongly suggest that lack of SOD leads to an oxygen-dependent increase in DNA damage, and that exonuclease III recognizes these DNA lesions and converts them to mutagenic lesions.

The lack of SOD seems to result in oxygen-dependent DNA damage, but the DNA lesions involved do not induce the SOS response. Furthermore, untargeted SOS mutagenesis—the mutagenic effect of SOS expression on undamaged DNA—is not a reflection of cryptic oxidative lesions in the DNA, since it is oxygen independent. It may well reflect lowered accuracy of DNA replication under conditions of SOS expression, as others have proposed (46).

The fact that cells lacking SOD show a significant increase in mutation frequency emphasizes the tremendous protective role SOD plays in mitigating such mutagenesis. The presence of SOD prevents most of the naturally occurring O_2 radicals from causing DNA damage. Given the apparent mutagenicity of $O_2^{\cdot-}$-dependent reactants, however, it is probable that bursts of oxidative stress, such as occur on reperfusion of ischemic tissue or exposure to oxidative mutagens, present a powerful threat to the genetic integrity of the cell. Although work has been further extended in *E. coli*, there exists evidence for oxidative mutagenesis in eukaryotes as well (e.g., see refs. 47 and 48). It has been suggested that active oxygen species may be the single most important agent involved in aging and cancer (49). Our work shows that an increase in the flux of superoxide radicals can indeed lead to increased mutagenesis.

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