Oxygen-dependent mutagenesis in \textit{Escherichia coli} lacking superoxide dismutase
(oxygen radicals/DNA lesions/exonuclease III)

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\textbf{ABSTRACT} \textit{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, \(O_2^-\) (1, 2). The reactivity of \(O_2\) radicals has been questioned (3, 4), although there is increasing evidence that \(O_2^-\) can directly damage macromolecules (5, 6). In addition, \(O_2^-\) readily undergoes further reduction to form \(H_2O_2\) and OH radicals, both of which are damaging to macromolecules, including DNA (7, 8). Further evidence suggesting that \(O_2^-\) 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 \textit{Escherichia coli} contains two forms of SOD, manganese SOD (MnSOD) encoded by the \textit{sodA} gene, and iron SOD (FeSOD) encoded by the \textit{sodB} gene (9, 10). \textit{sodB} is constitutive and \textit{sodA} is inducible under increased levels of \(O_2\) (11, 12). Hyperbaric oxygen has been shown to be mutagenic and toxic to wild-type \textit{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 \textit{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 \textit{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 \textit{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.

\textbf{MATERIALS AND METHODS}

\textbf{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). Pl transductions and transformation with plasmids were performed as described elsewhere (26, 27). Antibiotics were used at the following concentrations: chloramphenicol, 10 \(\mu g/ml\); kanamycin, 40 \(\mu g/ml\); tetracycline, 12.5 \(\mu g/ml\); and ampicillin, 50 \(\mu g/ml\).

\textbf{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).

\textbf{Use of the sfiA::lac Fusion to Monitor SOS Induction.} Cells were lysogenized with \(\lambda\text{p(sfiA::lac)clind}\) as described (28). \(\beta\)-Galactosidase activity was measured according to Miller

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Abbreviation: SOD, superoxide dismutase.

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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 of a 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 O2− 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 O2 radicals. When
Table 3. Thy" → Thy" forward mutation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Thy&quot; mutants per 10^7 cells* plated on LBT plus trimethoprim</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>GC4468</td>
<td>Wild type</td>
<td>21</td>
</tr>
<tr>
<td>QC773</td>
<td>sodB</td>
<td>14</td>
</tr>
<tr>
<td>QC772</td>
<td>sodA</td>
<td>25</td>
</tr>
<tr>
<td>QC774</td>
<td>sodA sodB</td>
<td>121</td>
</tr>
<tr>
<td>QC774/pDT1.5</td>
<td>sodA sodB</td>
<td>19</td>
</tr>
<tr>
<td>QC778/HS1.8</td>
<td>sodA sodB</td>
<td>21</td>
</tr>
</tbody>
</table>

Thy" cells were grown in LB broth at 37°C aerobically, hyperoxygenated, or anaerobically to an OD<sub>600</sub> 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.

Table 4. Mutagenesis in recA strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Thy&quot; → Thy&quot;&lt;sup&gt;a&lt;/sup&gt; mutants per 10&lt;sup&gt;7&lt;/sup&gt; viable cells</th>
<th>Rif&quot; → Rif&quot;&lt;sup&gt;b&lt;/sup&gt; mutants per cell, per generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC973</td>
<td>recA</td>
<td>14</td>
<td>1.4 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>QC774</td>
<td>sodA sodB</td>
<td>87</td>
<td>2.8 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>QC905</td>
<td>sodA sodB</td>
<td>99</td>
<td>2.7 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 5. Tif-mediated mutagenesis with and without oxygen

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30°C</td>
<td>42°C</td>
</tr>
<tr>
<td>10</td>
<td>292</td>
<td>5</td>
<td>167</td>
</tr>
<tr>
<td>14</td>
<td>292</td>
<td>9</td>
<td>199</td>
</tr>
<tr>
<td>18</td>
<td>287</td>
<td>11</td>
<td>223</td>
</tr>
<tr>
<td>Average</td>
<td>290</td>
<td>8</td>
<td>196</td>
</tr>
</tbody>
</table>

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<sup>8</sup> cells/ml, washed, and plated (10<sup>7</sup> 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.

units/mg (dry wt), increasing to 2500 units/mg (dry wt) in the presence of naladixic acid at 40 μg/ml.

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) strain growing aerobically or anaerobically. The recA(Tif) mutation induces the SOS response spontaneously at 42°C (23), and the ftsZ(SifB) 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 activity.

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<sup>a</sup>Exonuclease III

<sup>b</sup>Rifampicin
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. 1A). 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. 1B). Thus, the presence of functional exonuclease III diminishes the effects of O₂ 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 O₂⁻ 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 O₂⁻ 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.

**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 O₂ 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, H₂O₂, 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 O₂ radicals, and the enhanced mutagenesis in these mutants disappears when cells are grown anaerobically. We conclude that O₂ 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 O₂ (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](attachment:1.png)

**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 O2 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 full-length exonuclease III enhanced the growth of a sodA sodB mutant exposed to an increased flux of O2 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 O2 radicals from causing DNA damage. Given the apparent mutagenicity of O2-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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