Hydrogen peroxide effects in *Escherichia coli* cells*©*

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Key words: hydrogen peroxide, *Escherichia coli*, DNA repair

We analyzed DNA lesions produced by H₂O₂ under low iron conditions, the cross adaptive response and the synergistic lethal effect produced by iron chelator-o-phenanthroline, using different *Escherichia coli* mutants deficient in DNA repair mechanisms.

At normal iron levels the lesions produced by H₂O₂ are repaired mainly by the exonuclease III protein. Under low iron conditions we observed that the Fpg and UvrA proteins as well as SOS and OxyR systems participate in the repair of these lesions.

The lethal effect of H₂O₂ is strengthened by o-phenanthroline if both compounds are added simultaneously to the culture medium. This phenomenon was observed in the wild type cells and in the *zthA* mutant (hypersensitive to H₂O₂).

*E. coli* cells treated with low concentrations of H₂O₂ (micromolar) acquire resistance to different DNA damaging agents. Our results indicate also that pretreatment with high (millimolar) H₂O₂ concentrations protects cells against killing, by UV and this phenomenon is independent of the SOS system, but dependent on RecA and UvrA proteins. H₂O₂ induces protection against lethal and mutagenic effects of N-methyl-N'-nitro-N-nitosoguanidine (MNNG). H₂O₂ also protects the cells against killing by cumene hydroperoxide, possibly with the participation of Ahp protein.

The cellular response to oxidative stress poses an important problem since the succeeding univalent reduction of molecular oxygen to water in the respiration cycle produces


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Abbreviations: MNNG, N-methyl-N'-nitro-N-nitosoguanidine; DNA-SSB, DNA single-strand breaks; AP, spurinic sites.
three active intermediates: superoxide radical anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH'). These so-called reactive oxygen species are potent oxidants of lipids, proteins, and nucleic acids (Demple, 1991).

The participation of transition metals such as Cu$^+$ and Fe$^{2+}$ acting as reducing agents in the formation of OH' by the Fenton reaction and the Haber-Weiss cycle has been suggested previously (Simpson et al., 1988; Halliwell & Gutteridge, 1989):

\[
\text{O}_2^- + \text{Me}^{n+} \rightarrow \text{O}_2 + \text{Me}^{(n-1)} \quad \text{(eqn.1)}
\]

\[
\text{H}_2\text{O}_2 + \text{Me}^{(n+1)} \rightarrow \text{OH}^- + \text{OH}^- + \text{Me}^n \quad \text{(eqn.2)}
\]

\[
\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2 \quad \text{(eqn.3)}
\]

In fact, a variety of metal chelators, as dipyrindyl, o-phenanthroline and desferroxamine, can block the Fenton reaction by occupying metal coordination sites and thus protecting the cells against the lethal effects of H$_2$O$_2$, confirming the role of iron in the generation of active oxygen species (Imlay & Linn, 1988).

Several studies indicate that killing of E. coli cells exposed to H$_2$O$_2$ is mainly due to damage to DNA (Hagensee & Moses, 1988; Imlay & Linn, 1988). In E. coli wild-type strains, the lesions induced by H$_2$O$_2$ are efficiently repaired due to the presence of specific repair enzymes: glycosylases, AP lyases and AP endonucleases (Myles & Sancar, 1989). Additionally, the recombinational repair is an important pathway in repairing DNA lesions produced by H$_2$O$_2$, the enhancement of RecA protein synthesis being the most important SOS response, since other components of the SOS system appear to be of little or no importance for recombination repair (Imlay & Linn, 1987).

Bacterial cells display an adaptive response to oxidizing agents, which means that exposure to low levels of H$_2$O$_2$ allows bacterial cells to survive exposure to further toxic doses of H$_2$O$_2$ (Demple & Hailbrook, 1983; Christman et al., 1985). This response is accompanied by the induction of at least 30 proteins (Morgan et al., 1986). The expression of nine of the proteins induced by H$_2$O$_2$ treatment is under the control of the oxyR gene (Demple, 1991). Several proteins, the expression of which is regulated by oxyR, have been identified, including catalase encoded by katG gene, and an alkyl hydroperoxide reductase (Ahp), encoded by ahpC and ahpF genes (Morgan et al., 1986; Storz et al., 1990). Moreover, E. coli cells exposed to low doses of H$_2$O$_2$ develop resistance to heat shock and ethanol (Jenkins et al., 1988), ultraviolet (UV) A (Tyrrel, 1985), formaldehyde (Nunoshiba et al., 1991), and menadione (Christman et al., 1985). This effect is known as the cross-adaptive response.

Over the last years we have been studying the effects of H$_2$O$_2$ in E. coli by analyzing the lesions produced under low iron conditions, the cross-adaptive response and the synergistic lethal effect produced by o-phenanthroline (Asad et al., 1995; Asad et al., 1994a; 1994b; Asad et al., 1997a; 1997b; Asad & Leitão, 1991).

MATERIAL AND METHODS

**Bacterial strains.** All the bacterial strains used in this work are listed in Table 1.

**Growth conditions.** Cells were grown as described by Asad & Leitão (1991).

**Survival to H$_2$O$_2$.** Cells in mid-exponential phase of growth were treated with 5 mM H$_2$O$_2$ for different time as described by Asad & Leitão (1991) or with different H$_2$O$_2$ concentrations for 20 min as described by Asad et al. (1995).

**Survival and mutation frequency of cells treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).** These experiments were performed as described by Asad et al. (1997b).

**Survival to UV (254 nm) and reactivation of λ phages.** These experiments were performed as described by Asad et al. (1994a).
Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Designation</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>F' thr-1 leuB6 thi-1 argE3 his-4 proA2 lacY1 galK2 xyl-5 ara-14 rpsL13 tax38 supE44</td>
</tr>
<tr>
<td>AB2463</td>
<td>As AB1157, but recA13</td>
</tr>
<tr>
<td>AB2494</td>
<td>As AB1157, but lexA1 (Ind-)</td>
</tr>
<tr>
<td>AB1884</td>
<td>As AB1157, but uvrC</td>
</tr>
<tr>
<td>AB1886</td>
<td>As AB1157, but uvrB</td>
</tr>
<tr>
<td>AB1886</td>
<td>As AB1157, but uvrA6</td>
</tr>
<tr>
<td>JC4728</td>
<td>As AB1157, but recA142</td>
</tr>
<tr>
<td>BH20</td>
<td>As AB1157, but fpg-1::KnR</td>
</tr>
<tr>
<td>BW9091</td>
<td>As AB1157, but xthA</td>
</tr>
<tr>
<td>KT211</td>
<td>As AB1157, but ogl</td>
</tr>
<tr>
<td>KT222</td>
<td>As AB1157, but ada</td>
</tr>
<tr>
<td>KT233</td>
<td>As AB1157, but ada-ogl</td>
</tr>
<tr>
<td>MS23</td>
<td>As AB1157, but alkA</td>
</tr>
<tr>
<td>MV1661</td>
<td>As AB1157, but aidB</td>
</tr>
<tr>
<td>RK4936</td>
<td>araD139 (argF-lac)205 fliB501 non-gyrA219 relA1 rpsL150 metE70 hisB::TN10</td>
</tr>
<tr>
<td>TA4112</td>
<td>As RK4936, but oxyA3</td>
</tr>
<tr>
<td>PQ65</td>
<td>F' thr-1 leuB6 thi-1 his-4 gyrD galE galY rpoB lacAU169 trpC::MudC Pho' sfiA::Mud(Ap lac)C</td>
</tr>
<tr>
<td>OG100</td>
<td>As PQ65, but ΔoxyR4 [ΔoxyR huiB]4</td>
</tr>
<tr>
<td>OG140</td>
<td>As PQ65, but ΔkatG3, katE12::Tn10</td>
</tr>
<tr>
<td>K12</td>
<td>wild type</td>
</tr>
<tr>
<td>TA4315</td>
<td>As K12, but akpA5</td>
</tr>
<tr>
<td>TA4321</td>
<td>As K12, but akpΔ5 (pNAQ10)</td>
</tr>
</tbody>
</table>

The AB strains were obtained from F. Howard-Flanders (University of Yale, New Haven, CT, U.S.A.). The RK4936 and TA4112 were obtained from Bruce Ames (University of California, Berkeley, CA, U.S.A.). The PQ65, OG100 and OG140 were obtained from P. Quillardet (Institut Pasteur, Paris, France). The BW9091 was obtained from Bernard Weiss (University of Michigan Medical School, MI, U.S.A.). The JC4728 was obtained from R. Devoret (Institut Curie, Orsay, France).

Survival to cumene hydroperoxide. Cells in the mid-exponential phase of growth were treated for 20 min with 2.5 mM H₂O₂ or without it at 37°C under shaking. The treatment was stopped by the addition of an excess of catalase (Sigma Chemical Co., St. Louis, MO, U.S.A.) which was added even to non-pretreated cells (100 units per ml) (Asad & Leitão, 1991). Cells were collected after centrifugation, resuspended in M9S medium (Miller, 1972), and exposed to different concentrations of cumene hydroperoxide (Sigma Chemical Co., St. Louis, MO, U.S.A.) previously dissolved in ethanol (50%).

Treatment with metal ion chelators. Cells in mid-exponential phase of growth were treated for 20 min with metal ion chelators as described by Asad & Leitão (1991). In the studies with simultaneous treatment with H₂O₂ and o-phenanthroline the procedure was performed as described by Asad et al. (1994b).

DNA sedimentation studies. The induction of DNA single-strand breaks (DNA-SSB) was followed by sedimentation through alkaline sucrose gradients by the procedure of MacGrath & Williams (1966) with slight modi-
fications that were described by Asad & Leitão (1991).

**SOS induction.** The induction of SOS system by H$_2$O$_2$ was measured in *E. coli* by means of a *sfi::lacZ* operon fusion according to the principle of SOS Chromotest described by Quillardet & Hofnung (1985).

**RESULTS AND DISCUSSION**

Effects of iron chelators in cells treated with H$_2$O$_2$

Survival of H$_2$O$_2$-treated *xthA* mutant

When the *xthA* mutant cells were pretreated with o-phenanthroline, dipyridyl or desferrioxamine for 20 min we observed that these iron chelators diminished the lethal effect induced by H$_2$O$_2$ (Fig. 1). A similar protective effect of o-phenanthroline, was observed in wild type (AB1157) and *polA* mutant (P3478) cells. However, a chelator of copper, neocuproine, did not inhibit the lethal effect of H$_2$O$_2$ (Asad & Leitão, 1991). Similar results were observed in mammalian cells (Mello-Filho & Meneghini, 1985).

These results point to participation of iron ions in the formation of lethal damages in bacterial cells, probably by formation of OH$^-$ from H$_2$O$_2$ through the Fenton reaction. The protection observed on pretreatment with o-phenanthroline and dipyridyl could be due to the capture of Fe$^{2+}$ ions resulting in the blockage of Fe-catalyzed H$_2$O$_2$ reduction to OH$^-$ (eqn. 2). Desferrioxamine protects the cells probably due to chelation of Fe$^{3+}$ ions, thus blocking their reduction to Fe$^{2+}$ (eqn. 1).

**DNA strand-break production induced by H$_2$O$_2$**

It is known that H$_2$O$_2$ treatment produces DNA single-strand breaks (DNA-SSB) *in vitro* and *in vivo* (Kobayashi *et al.*, 1990; Tachon, 1990; Hagensee & Moses, 1989). Supposing that such breaks are produced by OH$^-$ and that transition metal ion chelators block the production of such radicals, one could expect that pretreatment with metal ion chelators would protect the cells against the DNA breaks produced by H$_2$O$_2$. However, DNA-SSB analysis in alkaline sucrose gradients in *xthA* mutant (Table 2) did not confirm this supposition.

Since metal ion chelators protect the cells against lethal effects of H$_2$O$_2$ and block the production of OH$^-$, we asked whether the

![Figure 1. Survival of *Escherichia coli* BW9091 (*xthA*) mutant treated with metal ion chelators and H$_2$O$_2$.](image)

Cultures in the mid-exponential phase of growth were treated in M9S medium at 37°C with metal ion chelators and then exposed to H$_2$O$_2$ (5 mM) and: (●) 1 mM dipyridyl; (□) 100 μM desferrioxamine; (△) 100 μM o-phenanthroline; or (○) treated with H$_2$O$_2$ alone.

### Table 2. Number of DNA-SSB per chromosome

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA-SSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>0</td>
</tr>
<tr>
<td>Only H$_2$O$_2$</td>
<td>230</td>
</tr>
<tr>
<td>100 μM o-phenanthroline</td>
<td>186</td>
</tr>
<tr>
<td>100 μM desferrioxamine</td>
<td>165</td>
</tr>
<tr>
<td>1 mM dipyridyl</td>
<td>165</td>
</tr>
</tbody>
</table>

DNA-SSB were obtained with *xthA* mutant in alkaline sucrose gradient. Cells were pretreated with metal chelators for 20 min and then treated with 5 mM H$_2$O$_2$ for 20 min. Metal ions chelators *per se* did not cause any DNA-SSB.
DNA-SSB produced in the presence of both H$_2$O$_2$ and iron chelators could be repaired in the $xth$A mutant, since this mutant is defective in the repair of sugar damage and AP sites produced by OH$^-$ (Demple & Halbrook, 1983). In fact, a time-dependent decrease in the number of DNA-SSB was observed when the cells were incubated after H$_2$O$_2$ neutralization (excess of catalase) (Table 3). In this case we suggest that the lesions produced by H$_2$O$_2$ under low iron conditions did not consist of AP sites or breaks containing 3'phosphate ends or 3'-terminal sugar fragments. The latter kind of lesion needs for repair prior action of exonuclease III, the $xth$A gene product (Kow & Van Houten, 1990). In contrast, similar experiments performed with polA mutant indicate that there was a slower repair of DNA-SSB (Table 3). Our interpretation is that there are at least two pathways of the repair of DNA lesions induced by H$_2$O$_2$ in E. coli: one produced by H$_2$O$_2$ through the Fenton reaction, where lesions need $xth$A and polA gene products to be repaired; the other lesions are produced by an iron-independent pathway, and their repair does not require the $xth$A gene product but requires the polA gene product (Asad & Leitão, 1991).

Participation of $fpg$ and $uwr$A proteins in the repair of lesions produced by H$_2$O$_2$ under low iron conditions

In order to verify if the base excision repair and the nucleotide excision repair participate in the repair of DNA iron-independent lesions induced by H$_2$O$_2$, mutant strains lacking these proteins were treated with H$_2$O$_2$ either at normal or at low iron levels (1 mM dipyridyl). The wild-type strain treated with H$_2$O$_2$ concentrations higher than 15 mM, under low iron conditions, was more sensitive to lethal effects of H$_2$O$_2$ than in the presence of normal levels of this metal (Fig. 2). When the $fpg$, $nfo$, $nth$, $xth$A mutants (base excision repair) and $uwr$A, $uwr$B, $uwr$C mutants (nucleotide excision repair) were treated with different concentrations of H$_2$O$_2$, under normal iron conditions, the survival was similar to that of the wild-type strain. The $xth$A mutant is hypersensitive to H$_2$O$_2$ (Asad, 1994). Under low iron conditions the survival of the mutants was similar to that of the wild-type strain, except for the $fpg$ and the $uwr$A mutants which, at higher H$_2$O$_2$ concentrations, were more sensitive to H$_2$O$_2$ than the wild-type strain. (Fig. 3).

Table 3. Kinetics of repair of DNA single-strand breaks induced by o-phenanthroline

<table>
<thead>
<tr>
<th>Time of incubation after H$_2$O$_2$ neutralization</th>
<th>$xth$A mutant</th>
<th>polA mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>(min)</td>
<td>Number of DNA-SSB per chromosome</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>142</td>
<td>155</td>
</tr>
<tr>
<td>10</td>
<td>42</td>
<td>76</td>
</tr>
<tr>
<td>30</td>
<td>16</td>
<td>38</td>
</tr>
<tr>
<td>60</td>
<td>7</td>
<td>30</td>
</tr>
<tr>
<td>Untreated control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Cells were pretreated with 100 μM o-phenanthroline for 20 min and then treated with 5 mM H$_2$O$_2$ for additional 20 min. An excess of catalase (5 μg/ml) was then added and cells centrifuged.

Figure 2. Survival of E. coli AB1157 (wild type) cells treated with dipyridyl and H$_2$O$_2$.

Cultures were treated in M9 medium at 37°C with 1 mM dipyridyl for 20 min and then treated with different concentrations of H$_2$O$_2$ for 20 min (●) or treated with H$_2$O$_2$ alone for 20 min (○).
plasmids pFPG60 and pSST10, carrying genes coding for the Fpg and UvrA proteins, with strains BH20 and AB1886, respectively, confirmed the participation of fpg and uvrA gene products in the iron-independent repair of H$_2$O$_2$ induced lesions (Fig. 3).

The results obtained with fpg mutant indicate that the Fpg protein participates in the repair of lesions produced by reactive oxygen species, including lesions produced by H$_2$O$_2$ under low iron conditions. So, we suggest that H$_2$O$_2$ under low iron conditions can produce lesions that could be either imidazole ring-opened purines or lesions similar to that produced by methylene blue plus visible light, like 8-hydroxyguanine (Czechot et al., 1991).

The results obtained with the uvrB and uvrC mutants are difficult to explain, since these mutants are as sensitive to H$_2$O$_2$ under low iron conditions as the wild-type cells. The current model for the incision reaction by UvrABC complex in the nucleotide excision repair in lesions induced by UVC suggests that the UvrA protein functions as a DNA damage recognition factor and UvrB as a delivery protein (Orren & Sancar, 1989). Therefore, hypersensitivity of the uvrA mutants to H$_2$O$_2$ indicates that the UvrA protein is required for the repair of lesions produced by H$_2$O$_2$ under low iron conditions (Asad et al., 1995).

Role of SOS and OxyR systems in the repair of lesions induced by H$_2$O$_2$ under low iron conditions

We found that recA13 mutant pretreated with 1 mM dipiridyl was more sensitive than the wild-type strain (Fig. 4), indicating that the recA gene product is necessary to repair DNA lesions produced by H$_2$O$_2$ under low iron conditions. Since the recA gene product is responsible for SOS functions and DNA recombination, we evaluated which of these functions is involved in the repair of lesions induced by H$_2$O$_2$ under low iron conditions. Our results indicate that the sensitivity of the recA142 mutant, which is defective in DNA recombination but is able to promote self-cleavage of LexA repressor (Sassanfar & Roberts, 1990), is similar to that of the wild-type strain (Fig. 4). However, the lexA1(Ind−) mu-
tant, defective in SOS induction by virtue of yielding a LexA protein resistant to self-cleavage promoted by RecA (Carvalho & Leitão, 1984) is as sensitive to $H_2O_2$ under low iron conditions as recA13 mutant (Fig. 4). These data suggest that recombinational repair does not play a relevant role in repairing lesions caused by $H_2O_2$ under low iron conditions, although this repair mechanism comprises the major benefit of SOS induction under normal iron conditions (Imlay & Linn, 1987). On the other hand, the induction of the SOS response is vital in the repair of lesions induced by $H_2O_2$ under low iron conditions. Thus, the SOS-mediated protection appears to be due to other SOS functions such as increased DNA excision repair, since uvrA mutation confers sensitivity to $H_2O_2$ under low iron conditions (Fig. 3).

Since the OxyR protein activates some stress proteins that are induced in E. coli in response to $H_2O_2$ treatment, such as catalase (Christman et al., 1985), it can be supposed that oxyR deletion mutants of E. coli may be sensitive to $H_2O_2$, although Imlay & Linn (1986) have reported that such deletion mutants are not unusually sensitive to killing by $H_2O_2$. Our results show that at the normal iron levels the $\Delta$oxyR4 deletion mutant (OG100) is not abnormally sensitive to killing by $H_2O_2$, indeed, it is as resistant as the wild type strain (Asad et al., 1997a). Nevertheless, under low iron conditions, the $\Delta$oxyR mutant was more sensitive to $H_2O_2$ than the wild-type strain (Fig. 5). This higher sensitivity cannot be related to a decrease in catalase level, since catalase deficient mutants (katG katE) treated with $H_2O_2$ under low iron conditions were not abnormally sensitive (Fig. 5). These results suggest that other enzymes regulated by oxyR are more important than catalase for the protection against lethal effects of $H_2O_2$ under low iron conditions.

SOS induction by $H_2O_2$ under low iron conditions

The induction of the SOS system by $H_2O_2$ was evaluated by measuring the induction of $\beta$-galactosidase (Quilaret & Hofnung, 1985) in wild-type cells (Table 4) and $\Delta$oxyR mutant.

![Figure 4. Survival of Escherichia coli cells treated with dipyridyl and $H_2O_2$.](image)

Cultures were treated in M9S medium at 37°C with 1 mM dipyridyl for 20 min and then treated with different concentrations of $H_2O_2$ for 20 min. Cells: (■) wild type; (▲) recA; (○) recA142; (□) lexA1.

![Figure 5. Survival of E. coli PQ65 (wild type) and OG100 ($\Delta$oxyR4) cells treated with dipyridyl and $H_2O_2$.](image)

Cultures were treated in M9S medium at 37°C with 1 mM dipyridyl for 20 min and then treated with different concentrations of $H_2O_2$ for 20 min (open symbols) or treated with $H_2O_2$ alone for 20 min (closed symbols). Cells: (■ - □) wild-type; (▲ - △) oxyR; (○ - ○) katE katG.
Table 4. β-Galactosidase induction by H2O2 in E. coli

<table>
<thead>
<tr>
<th>H2O2 concentration (mM)</th>
<th>Wild type with dipyridyl</th>
<th>Wild type without dipyridyl</th>
<th>ΔoxyR mutant with dipyridyl</th>
<th>ΔoxyR mutant without dipyridyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>2.9</td>
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<tr>
<td>5</td>
<td>4</td>
<td>4</td>
<td>11.2</td>
<td>7.5</td>
</tr>
<tr>
<td>7.5</td>
<td>3</td>
<td>6</td>
<td>14.2</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>2.8</td>
<td>9.2</td>
<td>14.5</td>
<td>4</td>
</tr>
<tr>
<td>12.5</td>
<td>2.2</td>
<td>9</td>
<td>14</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Dose-response for the induction of the σ70:leucZ fusion in E. coli PQ85 (wild type) and Oxy100(ΔoxyR4) mutant treated with 1 mM dipyridyl and H2O2. The standard deviations did not exceed 8% at all points.

Our results show that the pattern of SOS induction by H2O2 under low iron conditions was distinct from that observed under normal iron conditions in wild-type cells. Under normal iron conditions, a significant induction was observed with 1 mM H2O2, while under low iron conditions the induction was only observed with 10 mM to 12.5 mM H2O2. On the other hand, the induction of β-galactosidase by H2O2 in the ΔoxyAR mutant was significantly higher than in the parental strain under normal iron conditions, indicating that these lesions were repaired after they have triggered the SOS response. On the other hand, under low iron conditions the induction was similar to that in the parental strain. So, the sensitivity of the ΔoxyAR mutants treated with H2O2 under low iron conditions (Fig. 5) was probably not due to DNA strand-breaks (Asad et al., 1997a).

**Synergistic lethal effect of H2O2 and o-phenanthroline**

In this study we observed a synergistic lethal effect when the xthA mutant cells were treated simultaneously with H2O2 and o-phenanthroline (Fig. 6). The same results were observed in wild type cells. The synergistic lethal effect was also observed when o-phenanthroline was added to the cultures before H2O2. Moreover, we observed that, in xthA mutants, there were about 435 DNA-SSB per chromosome after simultaneous treatment with H2O2 and o-phenanthroline, compared with about 230 DNA-SSB after treatment with H2O2 alone. These results indicate that a high number of DNA single-strand breaks is responsible, at least in part, for the increased lethality. On the other hand, after 5 min of prior

Figure 6. Survival of E. coli BW9091 (xthA) mutant treated with o-phenanthroline and H2O2.
Cultures were treated in M9S medium at 37°C with 100 M o-phenanthroline and then treated with indicate concentrations of H2O2 for 20 min. (□) Simultaneous treatment; (●) 20 min of prior treatment; (○) control H2O2 without o-phenanthroline.
treatment with this iron chelator, a protection against H\textsubscript{2}O\textsubscript{2} lethal effects was observed (Fig. 6).

This effect was caused by a Fe\textsuperscript{2+}–o-phenanthroline–H\textsubscript{2}O\textsubscript{2} complex, since when the xthA mutant was pretreated for 20 min with 1 mM dipyridyl and then exposed to H\textsubscript{2}O\textsubscript{2} and o-phenanthroline simultaneously, the synergistic lethal effect was inhibited.

It is known that the equilibrium of formation and dissociation of the Fe\textsuperscript{2+}–o-phenanthroline complex follows equations 4, 5 and 6 (Burgers & Prince, 1965):

\[
\begin{align*}
\text{Fe}^{2+} + \text{Ph} & \rightarrow \text{FePh}^{2+} & \text{(eqn. 4)} \\
\text{FePh}^{2+} + \text{Ph} & \rightarrow \text{FePh}_{2}^{2+} & \text{(eqn. 5)} \\
\text{FePh}_{2}^{2+} + \text{Ph} & \rightarrow \text{FePh}_{3}^{2+} & \text{(eqn. 6)} \\
\end{align*}
\]

Thus, our data suggest that, since the equilibria of the bis- and mono-complexes of Fe\textsuperscript{2+}–o-phenanthroline are rapidly established and since these bis- and mono-complexes react quickly with H\textsubscript{2}O\textsubscript{2} (Burgers & Prince, 1965), there H\textsubscript{2}O\textsubscript{2}-mono- and H\textsubscript{2}O\textsubscript{2}-bis complexes would be formed and they could be extremely lethal to the cells.

In this case, when the cells are treated simultaneously with H\textsubscript{2}O\textsubscript{2} and o-phenanthroline, H\textsubscript{2}O\textsubscript{2} would react with mono- and bis-complex before tris-complex formation. After 5 min of prior treatment with o-phenanthroline the tris-complex should be formed; then, all Fe\textsuperscript{2+} would be chelated, and in this case there could be no OH\textsuperscript{-} formation by H\textsubscript{2}O\textsubscript{2} through the Fenton reaction.

Therefore, our results could be explained by the different kinetics of reaction of o-phenanthroline with intracellular Fe\textsuperscript{2+} and by the fact that the mono- and bis-complexes are more reactive than the intracellular Fe\textsuperscript{2+} to produce OH\textsuperscript{-} (Asad et al., 1994b).

Cross adaptive response

UVC (254 nm)

Prior exposure of E. coli to low doses of H\textsubscript{2}O\textsubscript{2} exerted little or no effect on the resistance of these cells to UVC (Demple & Halbrook, 1983). However, pretreatment with

Figure 7. Effect of H\textsubscript{2}O\textsubscript{2} pretreatment on the UV resistance in AB1157 (wild type) (panel A) and AB2494 (lexA1) (panel B) strains.

 Cultures were incubated for 20 min in M9S medium containing 2.5 mM H\textsubscript{2}O\textsubscript{2} prior to UVC (254 nm) exposure. This concentration of H\textsubscript{2}O\textsubscript{2} reduced survival to about 50% under our experimental conditions. Cells: (□) control; (△) pretreated. Plots represent the mean of three experiments.
higher H$_2$O$_2$ concentration (2.5 mM) protected wild-type cells against UVC-irradiation (Fig. 7A). This protection is independent of the SOS response, since it was also observed in a lexA1 mutant (Fig. 7B), and is dependent on DNA excision repair, since this protection was not observed in uvrA mutant (Asad et al., 1994a). Since H$_2$O$_2$ causes a cell division delay even after its removal (Imley & Linn, 1987), one can explain 2.5 mM H$_2$O$_2$-mediated protection by the increase in the duration of the post-irradiation cell growth inhibition period caused by UVC, which would allow pre-existing repair enzymes to act longer. On the other hand, DNA repair activities other than those known to be involved in SOS-response could be induced during H$_2$O$_2$ pretreatment. This possibility was verified by examining the ability of H$_2$O$_2$-pretreated cells to repair incoming damaged DNA. In this way, experiments similar to those described by Weigle (1953), but involving treatment of the cells with 2.5 mM H$_2$O$_2$ instead of UVC, were carried out with UVC irradiated phages.

The results show an enhanced survival of UV-irradiated phages in the wild-type cells treated with 2.5 mM H$_2$O$_2$, as compared to the untreated ones (Table 5).

Similar results were observed with the lexA1 mutant, indicating that this response is SOS independent. These results are in contrast to the lack of reactivation of UVC-damaged phage in UVC-irradiated lexA (Ind$^-$) mutant (Carvalho & Leitão, 1984). However, this response is dependent on RecA and UvrA proteins, since there was no UVC-damaged phage reactivation in recA and uvrA mutant cells pretreated with 2.5 mM H$_2$O$_2$. The oxyA3 mutant, in which the adaptive response to H$_2$O$_2$ does not occur (Imley & Linn, 1987), presented results similar to those observed in wild type and lexA1 mutant, indicating that the oxyR regulon does not participate in the UVC-damaged phage repair which takes place in H$_2$O$_2$-treated cells.

Our results suggest that H$_2$O$_2$ could induce an additional system (besides SOS) that is not under LexA control, but is dependent on the activation of RecA. In this case, UvrA protein plays a role in DNA repair of UVC-damaged bacteriophage.

N-methylN'-nitro-N-nitrosoguanidine

Demple & Halbrook (1983) reported that prior exposure of E. coli to low doses of H$_2$O$_2$

| Table 5. Reactivation of UV-irradiated phages in H$_2$O$_2$-treated E. coli cells |
|---------------------------------|-------------------|
| Strain (genotype) | Reactivation factor |
| AB1157 (wild type) | 15 |
| AB2494 (lexA1) | 12 |
| AB2463 (recA19) | 0.6 |
| AB1886 (uvrA6) | 0.5 |
| TA1112 (oxyD3) | 10 |

Phage survival was quantified by the reactivation factor, which is the ratio between phage survival in H$_2$O$_2$-treated and nontreated cells. Phage survival in nontreated cells was about 0.01% in all strains. The experiments were carried out in triplicate and the results are the average mean of 3 independent experiments. The standard deviation did not exceed 30%.

![Figure 8. Survival of E. coli AB1157 (wild type) cells treated with MNNG.](image)

Cultures were pretreated in M9S medium at 37°C for 20 min with 5 mM H$_2$O$_2$ (open symbols) or without it (■). Then the cells were treated with different concentrations of MNNG for 20 min. (□) MNNG added immediately, (○) MNNG added after 30 min incubation in M9S.
has no effect on the resistance of these cells to alkylating agents. However, pretreatment with a higher H$_2$O$_2$ concentration (5 mM) protects the wild-type strain (AB1157) against the lethal effects of MNNG. This protecting effect decreases after 15 min and is drastically reduced 30 min after the end of H$_2$O$_2$ treatment (Fig. 8). H$_2$O$_2$ protection against the effect of MNNG does not seem to be dependent on de novo protein synthesis, since the addition of chloramphenicol (200 µg/ml) 30 min before treatment with H$_2$O$_2$, does not interfere with the protection. Cells pretreated with 5 mM H$_2$O$_2$ and treated with MNU and ENU also exhibit protection against lethal effect of these agents (not shown). We found that 5 mM H$_2$O$_2$ also protected ada, ogt, ada-ogt, alkA and aidB mutants against the lethal effect of MNNG (Asad et al., 1997b).

Mutation frequency induced by MNNG is reduced by H$_2$O$_2$ pretreatment in the wild-type cells (Fig. 9) and in the ogt mutant (not shown). In contrast, this reduction is not observed in the ada mutant (not shown).

Since both the distribution and reparability of alkylation damage are influenced by DNA context (Horsfall et al., 1990), our results could be explained by the fact that the DNA alkylation pattern induced by MNNG can be altered when the DNA has already been oxidized. One possibility to explain the reduction of the Ada-dependent mutagenic effect of MNNG by pre-treatment of cells with H$_2$O$_2$ is that the DNA sequences altered by H$_2$O$_2$ could improve the interactions of Ada protein with methylated DNA and accelerate removal of mutagenic lesions induced by MNNG. In fact, there is evidence that neighboring nucleotides can influence the activity of alkyltransferase (Horsfall et al., 1990; Klein & Oesch, 1990).

Cumene hydroperoxide

Our results show that 2.5 mM H$_2$O$_2$ pretreatment protects wild-type cells against killing by cumene hydroperoxide (Fig. 10A). This protection was independent of the OxyR system, since it was also observed in the TA4112 (oxyA3) mutant (not shown). Furthermore, this response did not require novel protein synthesis, since it was observed in wild-type cells and oxyA3 mutants treated with chloramphenicol (160 µg/ml) for 30 min prior to H$_2$O$_2$ treatment (not shown). On the other hand, in the ahp deletion mutant cells (TA4315 ahpΔ5) no protection was observed indicating that Ahp protein plays a major role in this response. When a cosmid containing the ahp gene was introduced into the deletion mutant, we observed that in this strain (TA4321 ahpΔ5 pAQ10) the protection conferred by the H$_2$O$_2$ pretreatment was restored (Fig. 10B) (Asad et al., 1998).

On the other hand, oxyR mutant is more sensitive to cumene hydroperoxide than ahp mutant, suggesting that there is another oxyR-dependent activity acting on cumene hydroperoxide-induced damage (Asad et al., 1998).

Our results indicate that a high H$_2$O$_2$ concentration can induce protection against UV

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**Figure 9. Mutagenesis of E. coli AB1157 (wild type) treated with MNNG.**

Cultures were pretreated in M9S medium at 37°C for 20 min with 5 mM H$_2$O$_2$ (open symbols) or without it (■). Then the cells were treated with different concentrations of MNNG for 20 min, and ArgE mutants were scored, (□) MNNG added immediately, (○) MNNG added after 30 min of incubation in M9S.
Cumene hydroperoxide (mM)

Figure 10. Survival of E. coli K-12 (wild type) (panel A), TA4315 (ahp) and TA4321 (ahpA5 pAQ10) (panel B) treated with cumene hydroperoxide.

Cultures were pretreated in M9S medium at 37°C for 20 min with 2.5 mM H₂O₂ (▲) or without it (■). Cells were then treated with different concentrations of cumene hydroperoxide for 20 min (▲, □) TA4321.

light (254 nm), MNNG and cumene hydroperoxide, independently of the adaptive response of the cells. Thus, these results indicate that high concentrations of H₂O₂ can induce cross-protection responses through various mechanisms that do not necessarily involve the induction of de novo protein synthesis.

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