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Hydrogen peroxide effects in *Escherichia coli* cells*°

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We analyzed DNA lesions produced by H_2O_2 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 H_2O_2 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 H_2O_2 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 *xthA* mutant (hypersensitive to H_2O_2).

***E. coli* cells treated with low concentrations of H_2O_2 (micromolar) acquire resistance to different DNA damaging agents. Our results indicate also that pretreatment with high (millimolar) H_2O_2 concentrations protects cells against killing, by UV and this phenomenon is independent of the SOS system, but dependent on RecA and UvrA proteins. H_2O_2 induces protection against lethal and mutagenic effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). H_2O_2 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*-nitrosoguanidine; DNA-SSB, DNA single-strand breaks; AP, apurinic sites.

three active intermediates: superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). 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 $OH^{\cdot}$ by the Fenton reaction and the Haber-Weiss cycle has been suggested previously (Simpson *et al.*, 1988; Halliwell & Gutteridge, 1989):

$$O_2^{\cdot-} + Me^{n+} \rightarrow O_2 + Me^{(n+1)} \quad \text{(eqn.1)}$$

$$H_2O_2 + Me^{(n+1)} \rightarrow OH^{-} + OH^{\cdot} + Me^{n+} \quad \text{(eqn.2)}$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{-} + OH^{\cdot} + O_2 \quad \text{(eqn.3)}$$

In fact, a variety of metal chelators, as dipyridyl, *o*-phenanthroline and desferrioxamine, can block the Fenton reaction by occupying metal coordination sites and thus protecting the cells against the lethal effects of H_2O_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_2O_2 is mainly due to damage to DNA (Hagensee & Moses, 1989; Imlay & Linn, 1988). In *E. coli* wild-type strains, the lesions induced by H_2O_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_2O_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_2O_2 allows bacterial cells to survive exposure to further toxic doses of H_2O_2 (Demple & Halbrook, 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_2O_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_2O_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_2O_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_2O_2. Cells in mid-exponential phase of growth were treated with 5 mM H_2O_2 for different time as described by Asad & Leitão (1991) or with different H_2O_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

Designation	Genotype
AB1157	F^- thr-1 leuB6 thi-1 argE3 his-4 proA2 lacY1 galK2 xyl-5 ara-14 rspL13 tsx-33 supE44
AB2463	As AB1157, but *recA13*
AB2494	As AB1157, but *lexA1* (*Ind*$^-$)
AB1884	As AB1157, but *uvrC*
AB1885	As AB1157, but *uvrB*
AB1886	As AB1157, but *uvrA6*
JC4728	As AB1157, but *recA142*
BH20	As AB1157, but *fpg-1*::Kn^R
BW9091	As AB1157, but *xthA*
KT211	As AB1157, but *ogt*
KT222	As AB1157, but *ada*
KT233	As AB1157, but *ada-ogt*
MS23	As AB1157, but *alkA*
MV1561	As AB1157, but *aidB*
RK4936	araD139 (argF-lac)205 flB5301 non-gyrA219 relA1 rpsL150 metE70 btuB::TN10
TA4112	As RK4936, but *oxyΔ3*
PQ65	F^- thr-1 leuB6 thi-1 his-4 pyrD galE galY rpoB lacΔU169 trp::MuC$^+$ Pho^c sfiA::Mud(Ap lac)C^{ts}
OG100	As PQ65, but Δ*oxyR4* [Δ(oxyR btuB)4]
OG140	As PQ65, but Δ*katG3*, *katE12*::Tn10
K12	wild type
TA4315	As K12, but *ahpΔ5*
TA4321	As K12, but *ahpΔ5* (pAQ10)

The AB strains were obtained from P. 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, Orsey, France).

Survival to cumene hydroperoxide. Cells in the mid-exponential phase of growth were treated for 20 min with 2.5 mM H_2O_2 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 H_2O_2 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_2O_2 was measured in *E. coli* by means of a *sfi::lacZ* operon fusion according to the principle of SOS Chromotest described by Quilardet & Hofnung (1985).

RESULTS AND DISCUSSION

Effects of iron chelators in cells treated with H_2O_2

Survival of H_2O_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_2O_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_2O_2 (Asad & Leitão, 1991). Similar results were observed in mammalian cells (Mello-Filho & Meneghini, 1985).

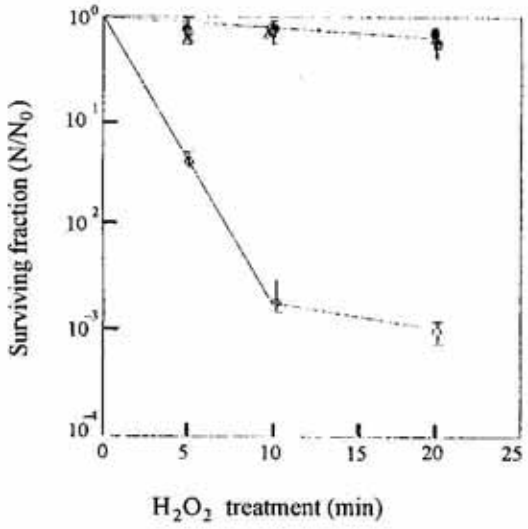

Figure 1. Survival of *Escherichia coli* BW9091 (*xthA*) mutant treated with metal ion chelators and H_2O_2.

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_2O_2 (5 mM) and: (●) 1 mM dipyridyl; (□) 100 μM desferrioxamine; (Δ) 100 μM *o*-phenanthroline; or (○) treated with H_2O_2 alone.

These results point to participation of iron ions in the formation of lethal damages in bacterial cells, probably by formation of $OH^{\cdot}$ from H_2O_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_2O_2 reduction to $OH^{\cdot}$ (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_2O_2

It is known that H_2O_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 $OH^{\cdot}$ 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_2O_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_2O_2 and block the production of $OH^{\cdot}$, we asked whether the

Table 2. Number of DNA-SSB per chromosome

Treatment	DNA-SSB
Untreated control	0
Only H_2O_2	230
100 μM *o*-phenanthroline	186
100 μM desferrioxamine	165
1 mM dipyridyl	195

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_2O_2 for 20 min. Metal ions chelators *per se* did not cause any DNA-SSB.

DNA-SSB produced in the presence of both H_2O_2 and iron chelators could be repaired in the *xthA* mutant, since this mutant is defective in the repair of sugar damage and AP sites produced by $OH^{\cdot}$ (Demple & Halbrook, 1983). In fact, a time-dependent decrease in the number of DNA-SSB was observed when the cells were incubated after H_2O_2 *neutralization* (excess of catalase) (Table 3). In this case we suggest that the lesions produced by H_2O_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 *xthA* 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_2O_2 in *E. coli*: one produced by H_2O_2 through the Fenton reaction, where lesions need *xthA* and *polA* gene products to be repaired; the other lesions are produced by an iron-independent pathway, and their repair does not require the *xthA* gene product but requires the *polA* gene product (Asad & Leitão, 1991).

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

Time of incubation after H_2O_2 neutralization (min)	*xthA* mutant	*polA* mutant
	Number of DNA-SSB per chromosome	
0	142	155
10	42	76
30	16	38
60	7	30
Untreated control	0	0

Cells were pretreated with 100 μM *o*-phenanthroline for 20 min and then treated with 5 mM H_2O_2 for additional 20 min. An excess of catalase (5 μg/ml) was then added and cells centrifuged.

Participation of *fpg* and *uvrA* proteins in the repair of lesions produced by H_2O_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_2O_2, mutant strains lacking these proteins were treated with H_2O_2 either at normal or at low iron levels (1 mM dipyridyl). The wild-type strain treated with H_2O_2 concentrations higher than 15 mM, under low iron conditions, was more sensitive to lethal effects of H_2O_2 than in the presence of normal levels of this metal (Fig. 2). When the *fpg*, *nfo*, *nth*, *xthA* mutants (base excision repair) and *uvrA*, *uvrB*, *uvrC* mutants (nucleotide excision repair) were treated with different concentrations of H_2O_2, under normal iron conditions, the survival was similar to that of the wild-type strain. The *xthA* mutant is hypersensitive to H_2O_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 *uvrA* mutants which, at higher H_2O_2 concentrations, were more sensitive to H_2O_2 than the wild-type strain, (Fig. 3). Complementation studies performed with

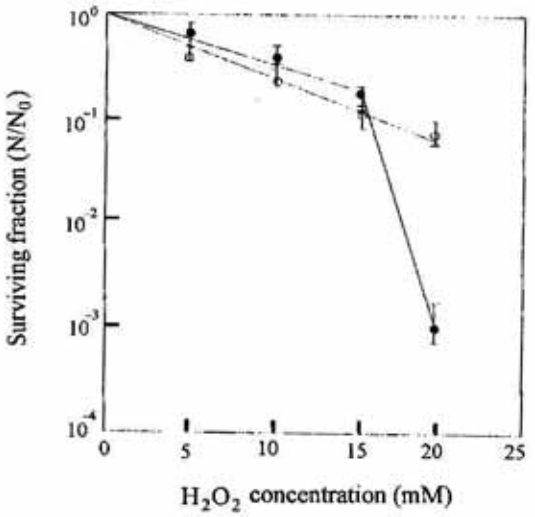

Figure 2. Survival of *E. coli* AB1157 (wild type) cells treated with dipyridyl and H_2O_2.

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 (●) or treated with H_2O_2 alone for 20 min (○).

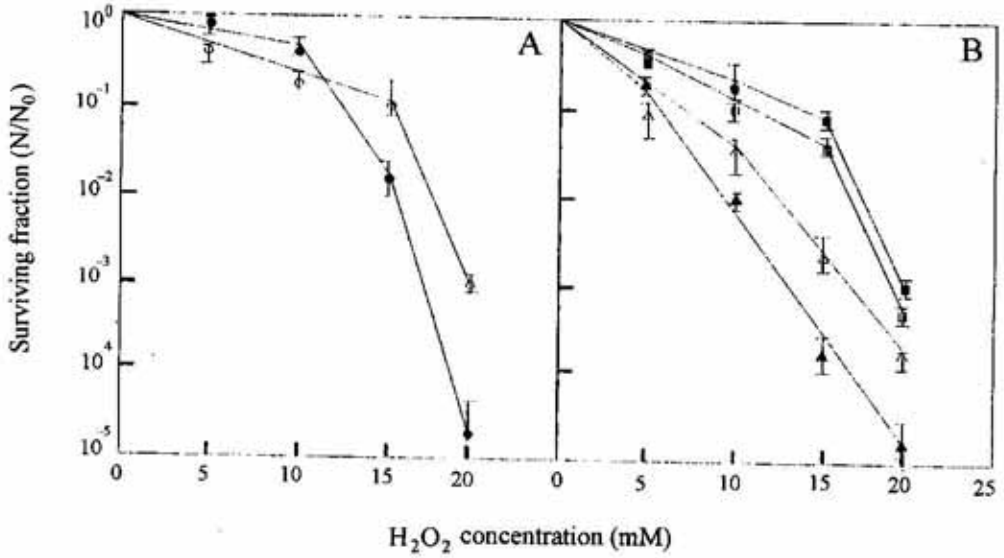

Figure 3. Survival of *E. coli* BH20 (*fpg*), AB1886 (*uvrA*), AB1885 (*uvrB*) and AB1884 (*uvrC*) cells treated with dipyridyl and H_2O_2.

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. Panel A, (●) *fpg* and (○) *fpg*/pFPG60. Panel B, (▲) *uvrA* and (△) *uvrA*/pSST10; (■) *uvrB*; (□) *uvrC*.

plasmids pFPG60 and pSST10, carrying genes coding for the Fpg and UrvA proteins, with strains BH20 and AB1886, respectively, confirmed the participation of *fpg* and *uvrA* gene products in the iron-independent repair of H_2O_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_2O_2 under low iron conditions. So, we suggest that H_2O_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 (Czeczot *et al.*, 1991).

The results obtained with the *uvrB* and *uvrC* mutants are difficult to explain, since these mutants are as sensitive to H_2O_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_2O_2 indicates that the UvrA protein is required for the repair of lesions produced by H_2O_2 under low iron conditions (Asad *et al.*, 1995).

Role of SOS and OxyR systems in the repair of lesions induced by H_2O_2 under low iron conditions

We found that *recA13* mutant pretreated with 1 mM dipyridyl 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_2O_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_2O_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 Δ*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 *oxyΔR* 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 *oxyΔR* are more important than catalase for the protection against lethal effects of H_2O_2 under low iron conditions.

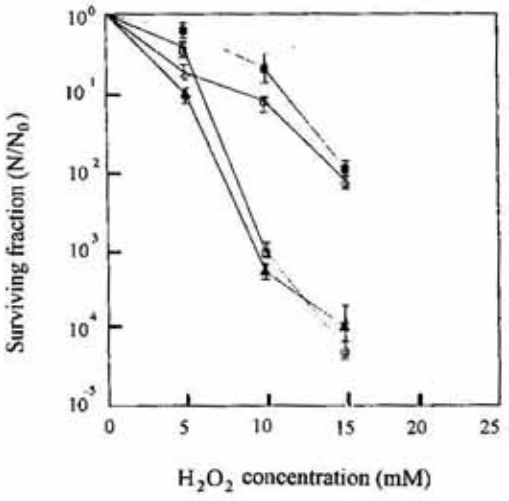

Figure 4. Survival of *Escherichia coli* cells treated with dipyridyl and H_2O_2.

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*.

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 β-galactosidase (Quilardet & Hofnung, 1985) in wild-type cells (Table 4) and *oxyΔR* mutant.

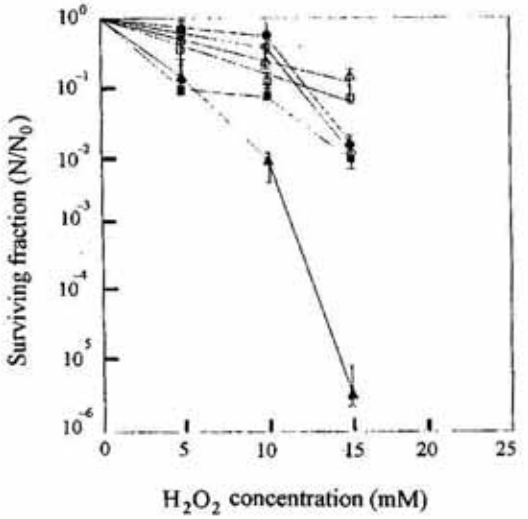

Figure 5. Survival of *E. coli* PQ65 (wild type) and OG100 (Δ*oxyR4*) cells treated with dipyridyl and H_2O_2.

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 H_2O_2 in *E. coli*

H_2O_2 concentration	Induction factor			
	Wild type		$\Delta oxyR$ mutant	
(mM)	with dipyridyl	without dipyridyl	with dipyridyl	without dipyridyl
0	1	1	1	1
1	10	2.9	19	3
2.5	4.5	2	21.5	3
5	4	4	11.2	7.5
7.5	3	6	14.2	12
10	2.8	9.2	14.5	4
12.5	2.2	9	14	2.8

Dose-response for the induction of the *sfiA::lacZ* fusion in *E. coli* PQ65 (wild type) and OG100($\Delta oxyR4$) mutant treated with 1 mM dipyridyl and H_2O_2. The standard deviations did not exceed 5% at all points.

Our results show that the pattern of SOS induction by H_2O_2 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 H_2O_2, while under low iron conditions the induction was only observed with 10 mM to 12.5 mM H_2O_2. On the other hand, the induction of β-galactosidase by H_2O_2 in the *oxy*ΔR 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 *oxy*ΔR mutants treated with H_2O_2 under low iron conditions (Fig. 5) was probably not due to DNA strand-breaks (Asad *et al.*, 1997a).

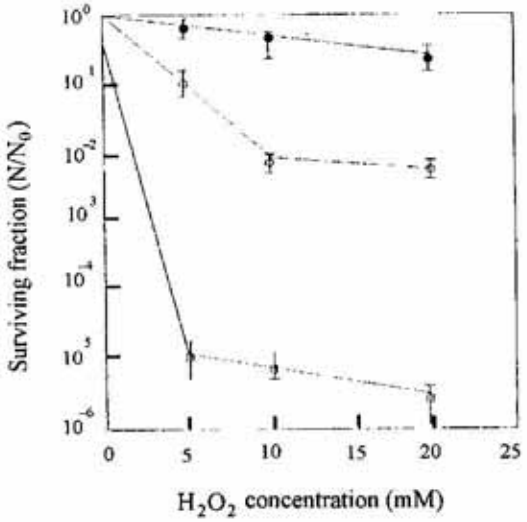

Figure 6. Survival of *E. coli* BW9091 (*xthA*) mutant treated with *o*-phenanthroline and H_2O_2.

Cultures were treated in M9S medium at 37°C with 100 M *o*-phenanthroline and then treated with indicate concentrations of H_2O_2 for 20 min. (□) Simultaneous treatment, (●) 20 min of prior treatment; (○) control H_2O_2 without *o*-phenanthroline.

Synergistic lethal effect of H_2O_2 and *o*-phenanthroline

In this study we observed a synergistic lethal effect when the *xthA* mutant cells were treated simultaneously with H_2O_2 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 H_2O_2. Moreover, we observed that, in *xthA* mutants, there were about 435 DNA-SSB per chromosome after simultaneous treatment with H_2O_2 and *o*-phenanthroline, compared with about 230 DNA-SSB after treatment with H_2O_2 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

treatment with this iron chelator, a protection against H_2O_2 lethal effects was observed (Fig. 6).

This effect was caused by a Fe^{2+}-*o*-phenanthroline-H_2O_2 complex, since when the *xthA* mutant was pretreated for 20 min with 1 mM dipyridyl and then exposed to H_2O_2 and *o*-phenanthroline simultaneously, the synergistic lethal effect was inhibited.

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

$$Fe^{2+} + Ph = FePh^{2+}$$

rapidly established equilibrium (eqn. 4)

$$FePh^{2+} + Ph = FePh_2^{2+}$$

rapidly established equilibrium (eqn. 5)

$$FePh_2^{2+} + Ph \rightarrow FePh_3^{2+}$$

rate-determining step (eqn. 6)

Thus, our data suggest that, since the equilibria of the bis- and mono-complexes of Fe^{2+}-*o*-phenanthroline are rapidly established and since these bis- and mono-complexes react quickly with H_2O_2 (Burgers & Prince, 1965), there H_2O_2-mono- and H_2O_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_2O_2 and *o*-phenanthroline, H_2O_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^{2+} would be chelated, and in this case there could be no $OH^{\cdot}$ formation by H_2O_2 through the Fenton reaction.

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

Cross adaptive response

UVC (254 nm)

Prior exposure of *E. coli* to low doses of H_2O_2 exerted little or no effect on the resistance of these cells to UVC (Demple & Halbrook, 1983). However, pretreatment with

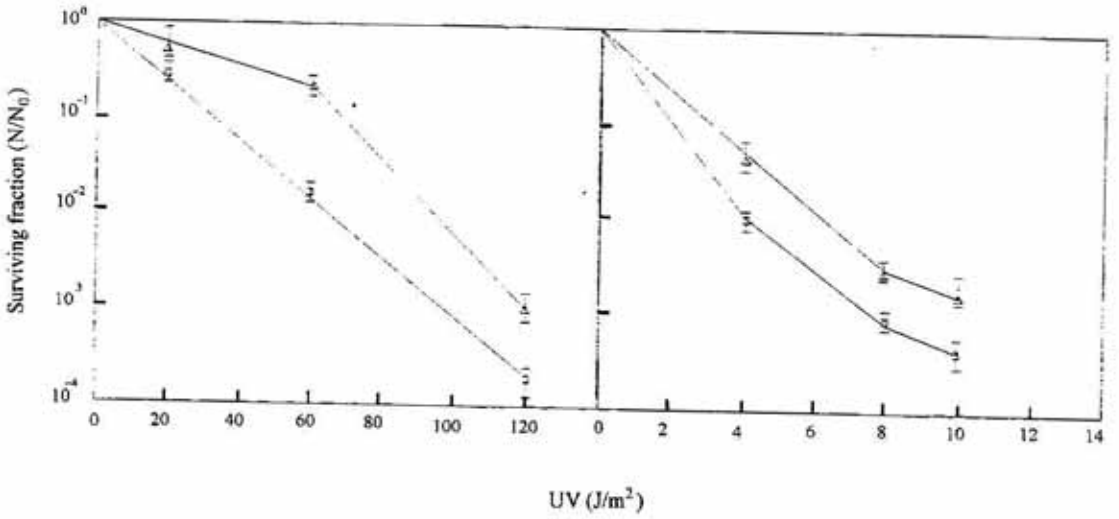

Figure 7. Effect of H_2O_2 pretreatment on the UV resistance in AB1157 (wild type) (panel A) and AB2494 (*lexA1*) (panel B) strains.

Cultures were incubated for 20 min in M9S medium containing 2.5 mM H_2O_2 prior to UVC (254 nm) exposure. This concentration of H_2O_2 reduced survival to about 50% under our experimental conditions. Cells: (□) control; (△) pretreated. Plots represent the mean of three experiments.

higher H_2O_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_2O_2 causes a cell division delay even after its removal (Imlay & Linn, 1987), one can explain 2.5 mM H_2O_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_2O_2 pretreatment. This possibility was verified by examining the ability of H_2O_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_2O_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_2O_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_2O_2. The *oxyΔ3* mutant, in which the adaptive response to H_2O_2 does not occur (Imlay & 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_2O_2-treated cells.

Our results suggest that H_2O_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.

Table 5. Reactivation of UV-irradiated phages in H_2O_2-treated *E. coli* cells

Strain (genotype)	Reactivation factor
AB1157 (wild type)	15
AB2494 (*lexA1*)	12
AB2463 (*recA13*)	0.6
AB1886 (*uvrA6*)	0.5
TA4112 (*oxyD3*)	10

Phage survival was quantified by the reactivation factor, which is the ratio between phage survival in H_2O_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 20%.

N-methyl-*N'*-nitro-*N*-nitrosoguanidine

Demple & Halbrook (1983) reported that prior exposure of *E. coli* to low doses of H_2O_2

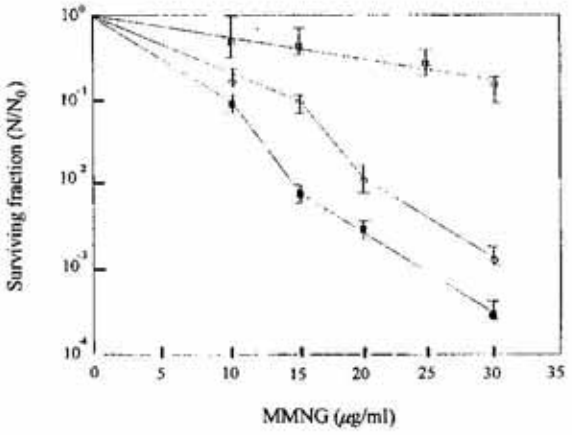

Figure 8. Survival of *E. coli* AB1157 (wild type) cells treated with MNNG.

Cultures were pretreated in M9S medium at 37°C for 20 min with 5 mM H_2O_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_2O_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_2O_2 treatment (Fig. 8). H_2O_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_2O_2, does not interfere with the protection. Cells pretreated with 5 mM H_2O_2 and treated with MNU and ENU also exhibit protection against lethal effect of these agents (not shown). We found that 5 mM H_2O_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_2O_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).

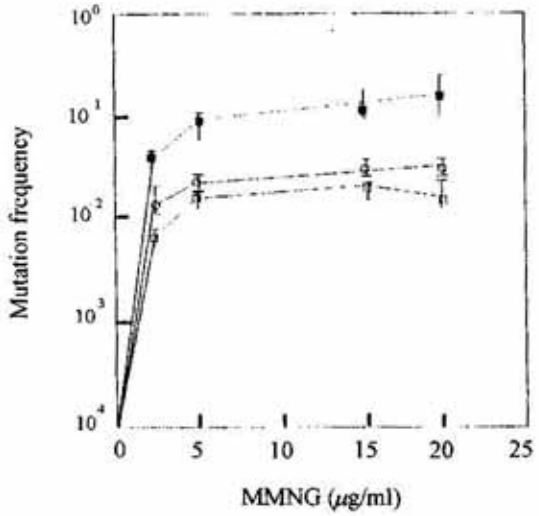

Figure 9. Mutagenesis of *E. coli* AB1157 (wild type) treated with MNNG.

Cultures were pretreated in M9S medium at 37°C for 20 min with 5 mM H_2O_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.

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_2O_2 is that the DNA sequences altered by H_2O_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_2O_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 (*oxy*Δ3) mutant (not shown). Furthermore, this response did not require novel protein synthesis, since it was observed in wild-type cells and *oxyΔ3* mutants treated with chloramphenicol (160 μg/ml) for 30 min prior to H_2O_2 treatment (not shown). On the other hand, in the *ahp* deletion mutant cells (TA4315 *ahp*Δ5) 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 *ahp*Δ5 pAQ10) the protection conferred by the H_2O_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_2O_2 concentration can induce protection against UV

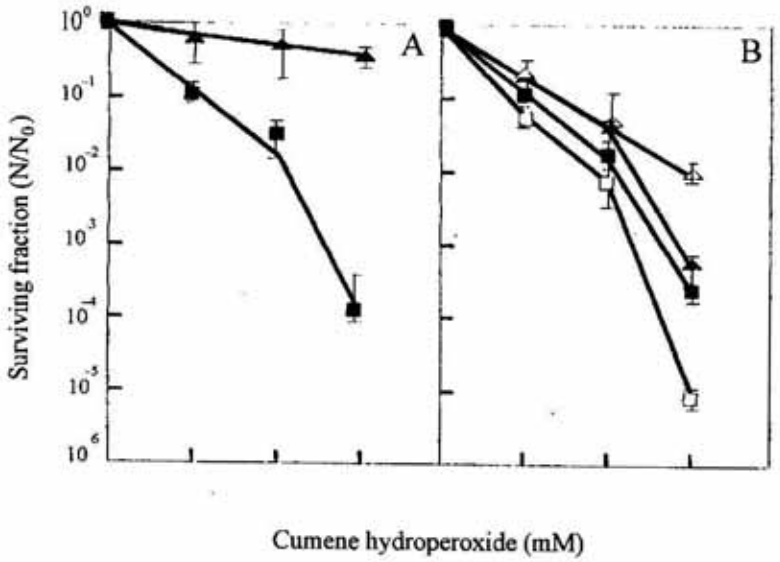

Figure 10. Survival of *E. coli* K-12 (wild type) (panel A), TA4315 (*ahp*) and TA4321 (*ahp*Δ5 pAQ10) (panel B) treated with cumene hydroperoxide.

Cultures were pretreated in M9S medium at 37°C for 20 min with 2.5 mM H_2O_2 (▲) 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 H_2O_2 can induce cross-protection responses through various mechanisms that do not necessarily involve the induction of *de novo* protein synthesis.

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