THE USE OF ALKALINE GEL ELECTROPHORESIS TO ANALYZE HYDROGEN PEROXIDE-CAUSED DNA DAMAGE AND REPAIR IN *ESCHERICHIA COLI*

by

Ross Eric Zirkle

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APPROVED:

Noel R. Krieg, Chairman

Brenda W. Shirley

Joseph O. Falkinham, III

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(ABSTRACT)

Reactive forms of oxygen such as hydrogen peroxide cause single-strand breaks in DNA. Most of the methods for estimating such breakage and subsequent repair are designed for eucaryotic cells and methods for use with bacteria are needed. Accordingly, a method based on alkaline gel electrophoresis of DNA was developed and tested with isogenic strains of *Escherichia coli* deficient in one or more DNA repair enzymes, viz., recA (*recA*), exonuclease III (*xthA*), DNA polymerase I (*polA*) or DNA polymerase I plus exonuclease III (*polA-xthA*). For DNA analysis of single-strand breaks, samples from a cell suspension were removed at 2 min intervals following an initial 15 min exposure to 20 mmol l^{-1} hydrogen peroxide. Catalase was added and the cells were embedded in blocks of low-melting point agarose and lysed to liberate their DNA . After alkaline gel electrophoresis, photographs of the gels were taken and the lengths of the distributions of DNA fragments were measured with a scanner and computer. The wild type and *recA* strain showed only a moderate increase in the length of the DNA distribution whereas the remaining strains all showed a large increase in the length of the distributions. The lengths of the distributions were correlated with cell survival at the same concentration of H_2O_2 and with the importance of particular DNA repair enzymes. Alkaline gel electrophoresis appears to be a relatively simple method for analyzing the level of H_2O_2-caused DNA damage and repair in *E. coli*.

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Introduction

Considerable interest has arisen recently in the area of oxidative damage to DNA and the repair of this damage. This interest stems in part from the realization that the uptake of oxygen by cells can be accompanied by the formation of reactive oxygen species, such as hydrogen peroxide and hydroxyl radicals, which can damage cellular components. Oxidative damage has been implicated in mutagenesis, carcinogenesis and aging. Reactive oxygen species have been shown to arise as by-products of cellular metabolism, as well as from spontaneous autooxidations or photochemical generations. One of the most important types of DNA damage that can be caused by reactive oxygen species is strand breaks. The repair of these strand breaks, as well as other kinds of oxidative damage to DNA, is important to prevent mutagensis and cell death. The development of multiple enzymatic pathways to repair damaged DNA in *Escherichia coli* is one of the indications of the importance of DNA repair.

Most of the methods currently used for the study of DNA strand breaks and their repair are useful for eucaryotes but are not applicable to bacteria. These methods depend on the structure of the eucaryotic nuclei or the relatively large size of eucaryotic cells to determine the amount of DNA damage or repair. DNA from the eucaryotic nucleus can be isolated while retaining some of its structure and the degree of unwinding that occurs to the nucleus is used to determine the amount of DNA damage. Alkaline elution is another common method used to assess DNA damage. This method requires filters with pore sizes too large for use with bacteria.

Some methods that are used to study DNA strand breaks and repair in bacteria require the use of plasmid DNA or enzymes and can only be used *in vitro*. The alkaline sucrose gradient method has been used with bacterial cells, but the DNA is subject to nonspecific sheering during cell lysis and

centrifugation, the method is time consuming and requires radiolabeled DNA, and statistical analysis is needed to interpret the results.

The goal of this research was to develop and test a relatively simple method that would be suitable for estimating the level of single-strand breakage in DNA and its subsequent repair following exposure of bacterial cells to hydrogen peroxide. In testing the method, we used *Escherichia coli* because of the detailed knowledge of DNA repair pathways in that species and because isogenic strains of *E. coli* were available that are deficient in specific repair enzymes.

Literature Review

Reactive Oxygen Species

Even though oxygen can be used by aerobes and microaerophiles as a terminal electron acceptor for respiration, it is also toxic to these organisms. Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{-\bullet}$), hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) react with and cause damage to cellular components. ROS may arise as byproducts of cellular metabolism as well as from spontaneous autooxidation or photochemical generation (Fridovich 1978).

Chemical properties of oxygen. Molecular oxygen is a biradical that contains two unpaired electrons one in each of its two π^* outer antibonding orbitals. Due to the parallel directions of the spin of these electrons, the reactivity of molecular oxygen is low. For oxygen to oxidize a non-radical molecule, the electrons to be removed from the molecule must also have parallel spin in order to be accepted in the antibonding orbitals. This requirement restricts the range of compounds oxidized by oxygen, since most compounds have pairs of electrons with opposite spins. The alternative to spontaneous two-electron reduction is a one-electron reduction that leads to formation of ROS. Singlet oxygen, which is also a ROS, is formed by the addition of energy to molecular oxygen. A scheme of sequential and one-electron reduction of molecular oxygen is shown in Figure 1.

Singlet oxygen. If an oxygen molecule is energized, a spin reversal of one of the π^* parallel electrons occurs, causing them to become antiparallel, but in different orbitals. This process produces sigma singlet oxygen ($^1\Sigma^+ O_2$). Sigma singlet oxygen is highly reactive but rapidly decays to delta singlet oxygen ($^1\Delta gO_2$) in which the two antibonding electrons occupy the same orbital. Singlet oxygen can cause lipid peroxidation by abstracting a hydrogen atom from a polyunsaturated lipid, followed by free radical propagation. Lipid peroxidation causes severe damage to the membrane structure, altering its fluidity and function (Aikens and Dix 1991).

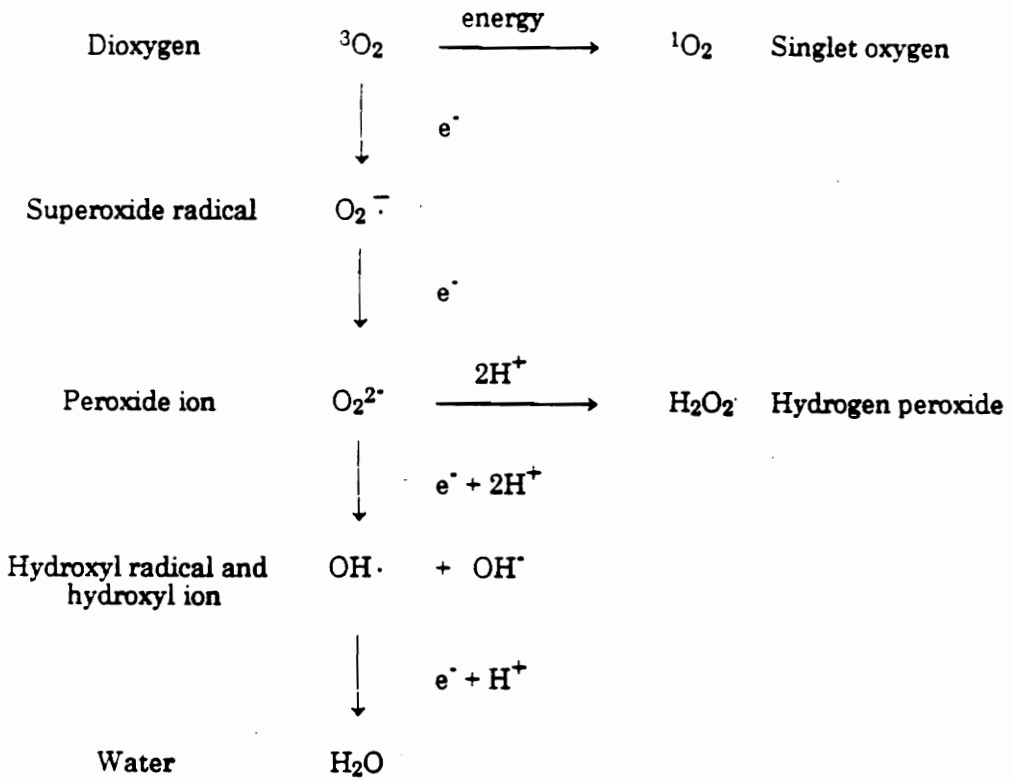


Figure 1. Derivation of reactive oxygen species from molecular oxygen.

Superoxide radicals. The superoxide radical is formed by the addition of one electron to molecular oxygen (reviewed by Fee and Valentine 1977). The superoxide radical can also exist in its protonated form as a perhydroxyl radical, but at physiological pH values it will dissociate to H^+ and $O_2^{-\bullet}$. The superoxide radical is unstable in aqueous solution and dismutates spontaneously, producing H_2O_2 and O_2. In this dismutation, one $O_2^{-\bullet}$ gives up its electron to another $O_2^{-\bullet}$:

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow O_2 + H_2O_2$$

Hydrogen peroxide. A two electron reduction of molecular oxygen produces the peroxide anion (O_2^{2-}) and at physiological pH the peroxide ion becomes immediately protonated to form H_2O_2. As stated earlier, a two electron reduction of oxygen is unusual. However, H_2O_2 can be generated by the aforementioned spontaneous superoxide radical dismutation. Hydrogen peroxide is not a free radical and yet it is very harmful to cells due to its ability to cross membranes (unlike $O_2^{-\bullet}$) and its function as a substrate for the Fenton reaction (reaction 2 below), which generates highly reactive hydroxyl radicals.

Hydroxyl radical. The three-electron reduction of oxygen produces oxyl anion radicals ($O^{-\bullet}$) and hydroxyl radicals. Because of its high pKa, $O^{-\bullet}$ may only exist in alkaline solution whereas at physiological pH it occurs as the hydroxyl radical ($OH^{\bullet}$), a very unstable and reactive oxidant (Czapski 1971). The hydroxyl radical may be produced by the reaction of hydrogen peroxide with a superoxide anion by the iron catalyzed Haber-Weiss reaction (reaction 3):

(1) $O_2^{-\bullet} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$

(2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$

(3) $H_2O_2 + O_2^{-\bullet} \rightarrow O_2 + OH^{\bullet} + OH^-$

In the absence of $O_2^{-\bullet}$, the addition of ferrous (Fe^{2+}) ions or the reduced ions of other transition metals such as Ti, Cr, Co, and Cu, to H_2O_2 can generate hydroxyl radicals by the Fenton reaction. The main function of $O_2^{-\bullet}$ is to reduce the metal ion that in turn donates its electron to H_2O_2, resulting in the production of $OH^{\bullet}$. Other reductants such as thiols, ascorbate, NADH, NADPH, and other radicals generated by redox-cycling drugs have been used *in vitro* to regenerate the reduced metal, thereby allowing the Fenton reaction to proceed (reaction 2) (Rowley and Halliwell 1983; Hetzberg and Dervan 1984) .

Reactions of ROS with Biological Systems

Determining the particular ROS that is directly responsible for a toxic effect is often difficult. For example, if a flux of $O_2^{-\bullet}$ causes cell death, this may be due to the production of H_2O_2 and $OH^{\bullet}$ from $O_2^{-\bullet}$ and not directly due to $O_2^{-\bullet}$ itself.

Hydroxyl radicals can cause strand breaks in DNA. Brawn and Fridovich (1980) showed that the aerobic action of xanthine oxidase on xanthine would cause strand scission in the DNA of the plasmid Col E1. Strand scission was demonstrated by migration differences of plasmid DNA in different conformations due to strand breakage after electrophoresis in a gel. Hydroxyl radicals were apparently the actual DNA-damaging oxidant because either catalase or SOD, as well as hydroxyl radicals scavengers such as mannitol and benzoate, prevented DNA strand scission. Kim *et al.* (1985) showed that enzymes could be cleaved by ROS that were produced in the presence of metal ions and reducing equivalents. Their results suggested that hydroxyl radicals are generated at specific sites on enzymes where iron is bound and are responsible for the inactivation and degradation of these enzymes.

Filho and Meneghini (1984) used alkaline sucrose gradients to demonstrate that phenanthroline and bipyridine, strong chelators of iron, protect DNA from single-strand breaks by H_2O_2 in human fibroblasts. This supports the concept that DNA single strand breaks are mediated by the Fenton reaction between Fe^{2+} and H_2O_2. Thiourea, an $OH^{\bullet}$ scavenger, also prevented the formation of single-stand breaks in fibroblasts.

Considerable attention has been given to the identification of the actual species that is responsible for strand scission in the reaction of H_2O_2 and DNA. Imlay *et al.* (1988) proposed that $OH^{\bullet}$ complexed to iron, as a Fenton reaction intermediate, produces a ferryl radical (Fe-$OH^{\bullet}$) and is the species responsible for DNA strand scission instead of free $OH^{\bullet}$. Sutton and Winterbourn (1989) suggested that higher oxidation states of a transition metal (tetravalent iron or trivalent copper) participate in causing DNA strand scission and other damage attributed to $OH^{\bullet}$. Walling (1975) proposed that when an iron chelator such as EDTA is present, some hydroxyl radicals may be trapped by the iron complex immediately after formation to create a "caged" reactive species. However, Progozelski *et al.* (1995) presented results consistent with the concept that $OH^{\bullet}$ is directly responsible for stand scission. Their data demonstrated that the DNA-cleavage patterns generated by a known source of hydroxyl radicals, viz., the γ-radiolysis of water, were identical to those generated by the reaction of $[Fe^{2+}(EDTA)]^{2-}$ with H_2O_2 in the presence of ascorbate.

Di Mascio *et al.* (1989) showed that singlet oxygen caused DNA strand breaks and loss of transforming activity in both plasmid and viral DNA. Blazek *et al.* (1989) detected single-stand breaks in pBR322 exposed to $^1O_2$. Devasagayam *et al.* (1991) also demonstrated that singlet oxygen, generated by thermal dissociation of the endoperoxied of 3,3'-(1,4-napthylidene) dipropionate, caused single-strand breaks in pBR332 DNA. An increase of plasmid DNA breakage was observed in the

presence of chemically-related sulfhydryl compounds, which can produce $OH^{\bullet}$ when reacted with molecular oxygen or $^1O_2$. Mannitol, a hydroxyl scavenger, decreased the amount of single-strand breaks in the plasmid DNA again suggesting the participation of $OH^{\bullet}$ in the breakage of DNA.

DNA Damage

Interaction of DNA with various physical or chemical agents can result in structural changes to DNA. These changes may lead to mutation or cell death. Oxidative damage has been a major area of investigation due to the generation of ROS by oxygen metabolism. Oxidative damage has also been implicated in the pathophysiology of a wide variety of diseases such as cancer, cardiovascular disease, neurological degeneration, immune dysfunction, and even aging (Ames *et al.* 1993).

Oxidative Damage to DNA. Attack by ROS is considered a major source of damage to DNA. As stated above, H_2O_2 and $O_2^{-\bullet}$ probably do not react with DNA directly and are important in the formation of $OH^{\bullet}$. Attack may occur either at the sugar or the base, leading to a variety of products. Rhaese *et al.* (1968) demonstrated that exposure of deoxynucleotides and deoxynucleosides to H_2O_2 causes the release and modification of all four bases. Dizdaroglu (1992) used gas chromatography/mass spectrometry to identify pyrimidine- and purine- modified DNA bases after exposure to hydroxyl radicals. Radical attack can also occur at the *N*-glycosylic bond and liberate an intact or modified base from the deoxyribose and generate an AP site. Examples of various types modified bases following radical exposure are illustrated in Figure 2. Blakely *et al.* (1990) identified many of the same modified bases in Figure 2 by gas chromatography-mass spectometry following the addition of H_2O_2 to an aqueous solution of calf thymus deoxyribonucleic acid.

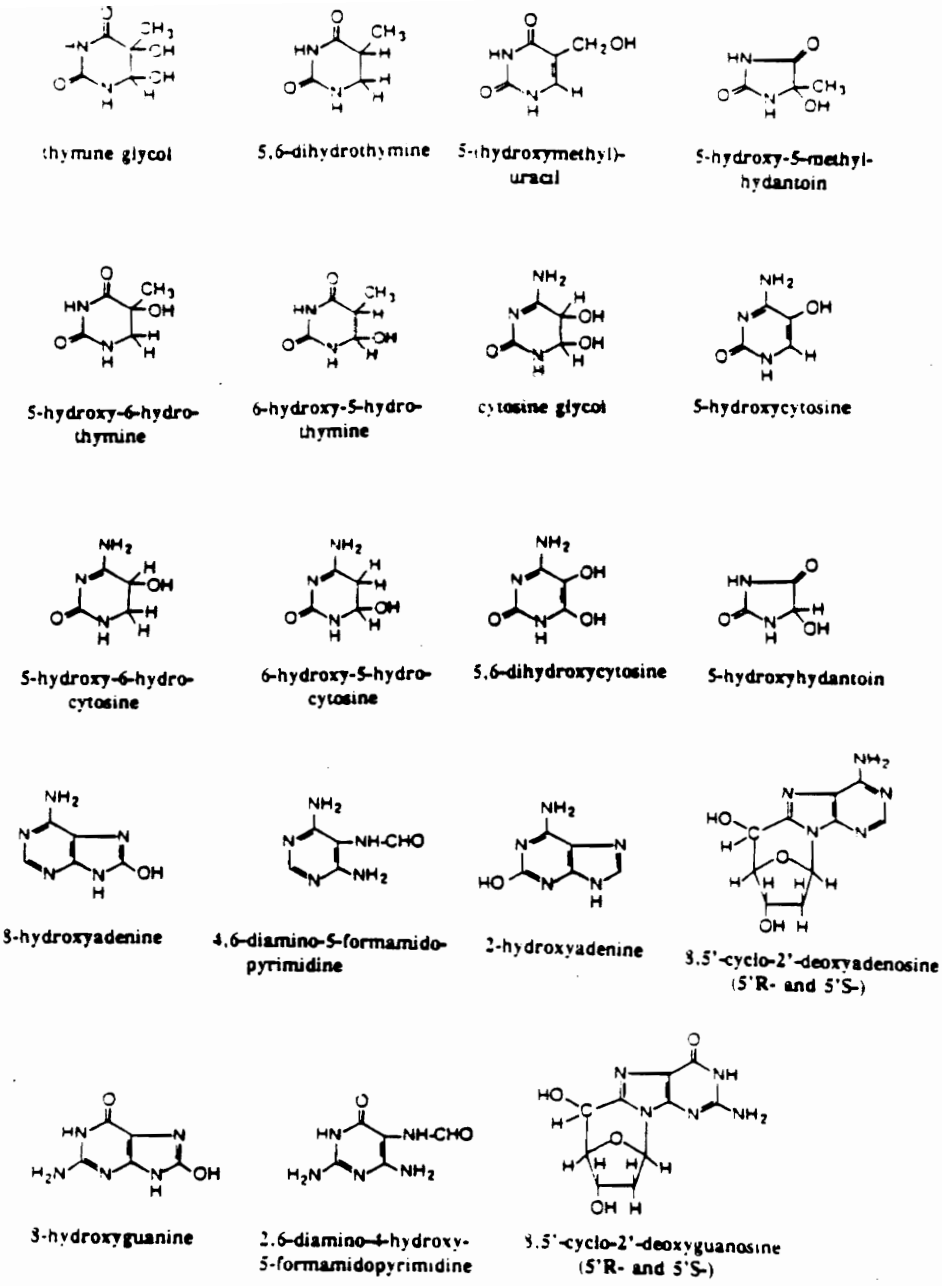


Figure 2. Examples of modified bases following hydroxyl radical exposure (From Dizarouglu 1992).

Rhaese and Freese (1968) first demonstrated the H_2O_2 caused strand breaks to DNA *in vitro* while Ananthaswamy and Eisenstrak (1977) showed that H_2O_2 caused single-strand breaks to DNA in *E. coli*. Attack on the deoxyribose of DNA leads to sugar fragmentation, base loss, and a strand break with a terminal sugar residue (Imlay and Linn 1988). Single-strand breaks are initiated by radical formation at the deoxyribose following the abstraction of a hydrogen atom by $OH^{\bullet}$ from any of the five carbon atoms (Dizdaroglu 1993). The sugar radical formed can undergo various reactions including the release of an altered or intact base, as well as strand breakage. The majority of these strand breaks are characterized by unusual or damaged termini which prevent their repair by a single DNA ligation step (Henner *et al.* 1982). Although the 5' end can retain a phosphate group, a 3'-OH group is usually not available which is required for DNA ligase in a single ligation step. This 3' termini often has a phosphate or phosphoglycolate group (Demple *et al.* 1986).

DNA Repair

The repair of different types of DNA damage is of utmost importance to the cell. DNA damage that is not repaired by the cell can lead to mutagenesis and cell death. Kuzminov (1995) proposed a model in which single-strand breaks, in either strand of DNA, cause a collapse of replication forks in *E. coli*. It has also been estimated that the human genome sustains as many as 10,000 oxidative "hits" per day per cell (Ames *et al.* 1993).

Base Excision Repair. DNA glycosylases are enzymes that initiate base excision repair by hydrolyzing the *N*-glycosylic bond of the modified or incorrect base to generate an AP site. A list of DNA glycosylases is shown in Table 1 as well as the specific substrates the glycosylases recognize. The removal of the AP sites is initiated by a second class of base excision enzymes called AP

Table 1. DNA Glycosylases. (From Friedberg et al. 1994)

Enzyme	Substrate	Products
Ura-DNA glycosylase	DNA containing uracil	Uracil + AP sites
Hmu-DNA glycosylase	DNA containing hydroxymethyluracil	Hydroxymethyluracil + AP sites
5-mC-DNA glycosylase	DNA containing 5-methylcytosine	5-methylcytosine + AP sites
Hx-DNA glycosylase	DNA containing hypoxanthine	Hypoxanthine + AP sites
Thymine mismatch-DNA glycosylase	DNA containing G·T mispairs	Thymine + AP sites
MutY-DNA glycosylase	DNA containing G·A mispairs	Adenine + AP sites
3-mA-DNA glycosylase I	DNA containing 3-methyladenine	3-Methyladenine + AP sites
3-mA-DNA glycosylase II	DNA containing 3-methyladenine, 7-methylguanine, or 3-methylguanine	3-Methyladenine, 7-methylguanine, or 3-methylguanine + AP sites
FaPy-DNA glycosylase	DNA containing formamidopyrimidine moieties, or 8-hydroxyguanine	2,6-Diamino-4-hydroxy-5-*N*-methylformamidopyrimidine and 8-hydroxyguanine + AP sites
5,6-HT-DNA glycosylase (endonuclease III)	DNA containing 5,6-hydrated thymine moieties	5,6-Dihydroxydihydrothymine or 5,6-dihydrothymine + AP sites
PD-DNA glycosylase	DNA containing pyrimidine dimers	Pyrimidine dimers in DNA with hydrolyzed 5′ glycosyl bonds + AP sites

endonucleases. These enzymes produce nicks in duplex DNA by hydrolyzing the phosphodiester bonds that are 5' or 3' to each AP site. Hydrolysis of the phosphodiester bond 5' to the AP site generates a 5' terminal deoxyribose-phosphate residue. The removal of this residue requires the participation of another group of enzymes called DNA-deoxyribophosphodiesterases (dRpase) which are not repair specific enzymes and can degrade free ends of DNA.

Besides the removal of modified or incorrect bases, some DNA glycosylases cause breakage of phosphodiester bonds *in vitro* (Friedberg *et al.* 1995). This associated AP lyase activity occurs via β-elimination 3' to the AP site and yields a 5'-terminal deoxynucleoside-5'phosphate and a 3' terminal unsaturated aldehyde (Wilde *et al.* 1989). These blocked 3' termini would require further processing by other enzymes to generate a 3' OH that can be used for repair synthesis.

An alternative mechanism for the incision of DNA at the AP site *in vivo* is the hydrolysis of the phosphodiester bonds catalyzed by an unassociated 5' AP endonuclease. This process would yield a 3' OH terminus and a 5' deoxyribose-phosphate terminus. The 3' OH terminus is a substrate for DNA polymerases that initiate DNA synthesis. Evidence suggests that this pathway probably operates during base excision repair *in vivo* (Friedberg *et al.* 1995). The two possible pathways of base excision repair are illustrated in Figure 3.

In *E. coli*, exonuclease III is one of the 5' AP endonucleases responsible for the incision event at the AP site. Exonuclease III was originally characterized as an exonuclease; its major physiological function as a 5' AP endonuclease was not discovered until after its name had become the accepted nomenclature. The activities of exonuclease III are inhibited by metal chelators, and are stimulated by 1-10 mM Mg^{2+}. Demple *et al.* (1983) showed that mutants lacking exonuclease III (*xth* strains) are hypersensitive to H_2O_2. The specific role of exonuclease III was determined by the analysis

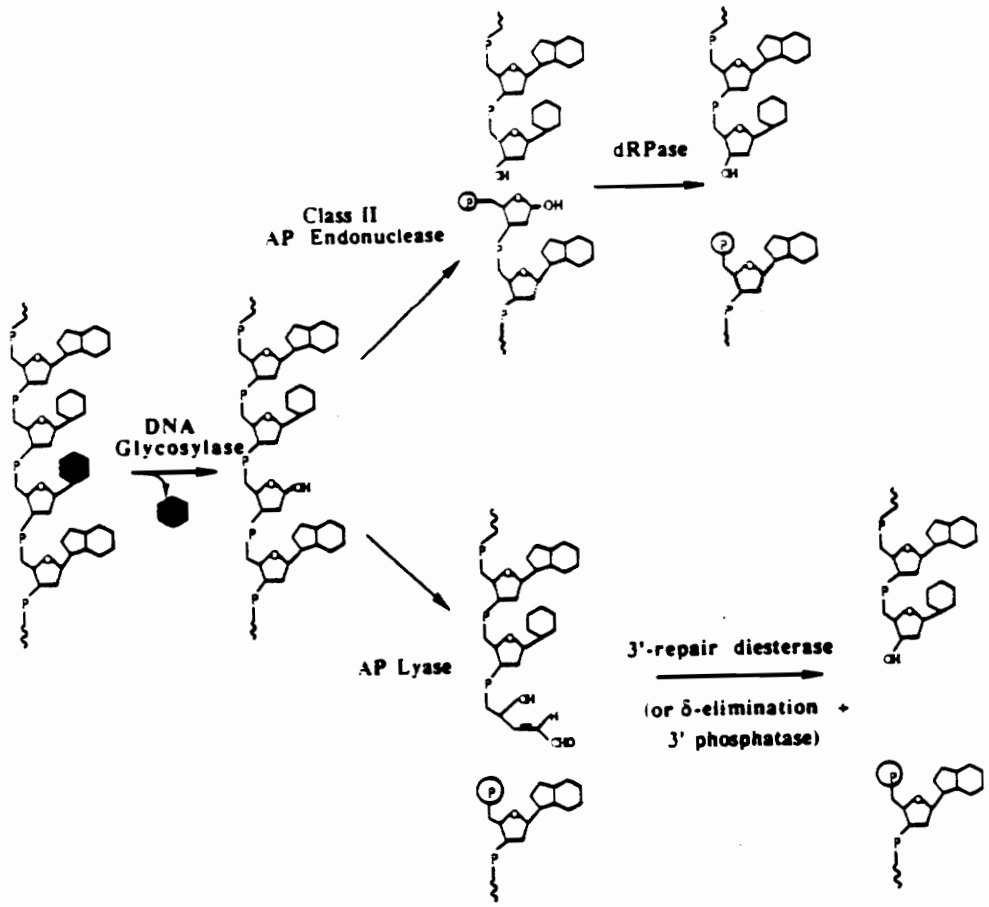


Figure 3. Two possible pathways of base excision repair (From Demple and Harrison 1994)

of chromosomal DNA from bacteria challenged with H_2O_2. Demple *et al.* (1986) demonstrated that the enzyme removes deoxyribose fragments from the 3'-termini of DNA strand breaks generated *in vivo* by treatment with H_2O_2. The role of exonuclease III has been established as both an AP endonuclease that is responsible for the excision event at a AP site, and an exonuclease that removes 3' phosphates or 3' phosphoglycolate esters from the 3'-termini of strand breaks due to radical attack (Demple *et al.* 1986; Demple and Harrison 1994). The removal of this 3' blocking provides substrates for DNA polymerase and DNA ligase for repair synthesis. Exonuclease III accounts for 99% of the 3' phosphatase activity present in *E. coli* (Demple *et al.* 1983).

In *E. coli*, repair of damage caused by ROS is considered to be carried out primary by base excision repair (Friedberg *et al.* 1995). The hypersensitivity to H_2O_2 that *xthA* mutants display indicate the importance of exonuclease III's role in the repair of oxidative damage to DNA. Demple *et al.* (1986) demonstrated that *xthA* mutants accumulated unrepaired DNA single-strand breaks with blocked 3' termini and concluded that an important function of exonuclease III is to restore 3' hydroxyl groups to act as primers for DNA polymerase I. Hagensee and Moses (1989) also showed *xthA* mutants can repair some single-strand breaks after H_2O_2 exposure but at a much more slower rate than wild type cells. They concluded that the delayed repair may be due to other enzymes that are not as efficient as exonuclease III.

Endonuclease IV is an enzyme that is another example of a 5' AP endonuclease (Ljungquist *et al.* 1976). Exonuclease III and endonuclease IV are responsible for ~95% and ~5% of total AP endonuclease activity in *E. coli*, respectively (Demple *et al.* 1986). Chemical agents such as paraquat and plumbagin, which are reduced by one-electron transfer reactions followed by auto-oxidation to generate superoxide radicals, induce the level of endonuclease IV (Chan and Weiss 1987).

Both the incision at the AP site and the removal of 3' blocking by AP endonucleases leave a 3' OH terminus and 5'-deoxyribose-phosphate terminus. The 3' OH is a primer-terminus for DNA polymerases in DNA synthesis but the completion of the repair process requires the removal of the 5'-deoxyribose-phosphate moieties. Franklin and Lidahl (1988) demonstrated an exonuclease in *E. coli* called DNA-deoxyribophosphodiesterase that carries out this function.

The interaction of the repair enzymes in base nucleotide excision repair is still uncertain. Hagensee and Moses (1989) have demonstrated at least two pathways of repair to H_2O_2-damaged DNA. Mutants strains of *E. coli* deficient in DNA polymerase I, DNA polymease III, and exonuclease III were used to determine cell survival and ability to repair DNA single-strand breaks after exposure to H_2O_2. DNA polymerase I mutants did not repair the single-strand breaks and they suggested that DNA polymerase I was required for the repair of H_2O_2 caused single-strand breaks. The other mutant strains demonstrated the ability to repair the single-strand breaks at different levels, indicating that multiple pathways are utilized to repair single-stand breaks. They concluded that one pathway would utilize exonuclease III, DNA polymerase III, and DNA polymerase I, while the other would utilize DNA polymerase I only.

Recently Zhang *et al.* (1992) investigated the interaction of base excision repair pathways that included *E. coli* strains deficient in DNA polymerase I, DNA polymerase III, exonuclease III, endonuclease IV, and an exonuclease III - endonuclease IV double mutant. They found that all the strains were able to repair the H_2O_2-caused damage to some degree and the levels of repair was different in each strain. They proposed that there were at least four pathways in *E. coli* to repair H_2O_2 damaged DNA; (1) DNA polymerase I; (2) exonuclease III/endonuclease IV and DNA polymerase I;

(3) exonuclease III/endonuclease IV and DNA polymerase III; and (4) a pathway that would utilize only endonuclease III/endonuclease IV.

Nucleotide Excision Repair. Nucleotide excision repair is a process by which damaged bases are enzymatically excised from DNA as oligonucleotides and the resulting gap filled in by repair synthesis and DNA ligase. In *E. coli*, the UrvA, UrvB and UrvC proteins act in a series of steps to recognize and bind to the damaged site, followed by incisions on both sides of the damage. DNA helicase, polymerase and ligase reactions then eliminate the damaged 12- or 13-mer oligonucleotide and complete the repair. The pathway of nucleotide excision repair is illustrated in Figure 4.

The exact mechanism by which the UrvABC complex is able to recognize and repair a wide variety of DNA lesions is not known. Van Houten (1990) suggests that recognition of damage is due to distortion of the DNA helix. *In vitro* studies have shown that some types of damage not considered distortive to the DNA helix are substrates for *E. coli* UrvABC endonuclease while some mismatches, loops caused by mispairing and naturally bent DNA are not substrates for the UrvABC endonuclease (Selby and Sancar 1990).

The *E. coli* UrvABC system is not pivotal for handling oxidative DNA damage and it is considered to be merely a secondary defense against oxidative damage (Demple and Harrison 1994). Imlay and Linn (1987) demonstrated that mutations in *urvA*, *uvrB* and other nucleotide excision repair genes did not confer abnormal sensitivity to killing by H_2O_2. Also, the UvrABC system can repair oxidative damage, such as thymine glycol and AP sites at relativity slow rates, but strand breaks appear not to be substrates for nucleotide excision repair. Hagensee and Moses (1989), using a *recA* mutant, also found no direct role of the RecA protein in the repair of H_2O_2-damaged DNA. The *lexA*-

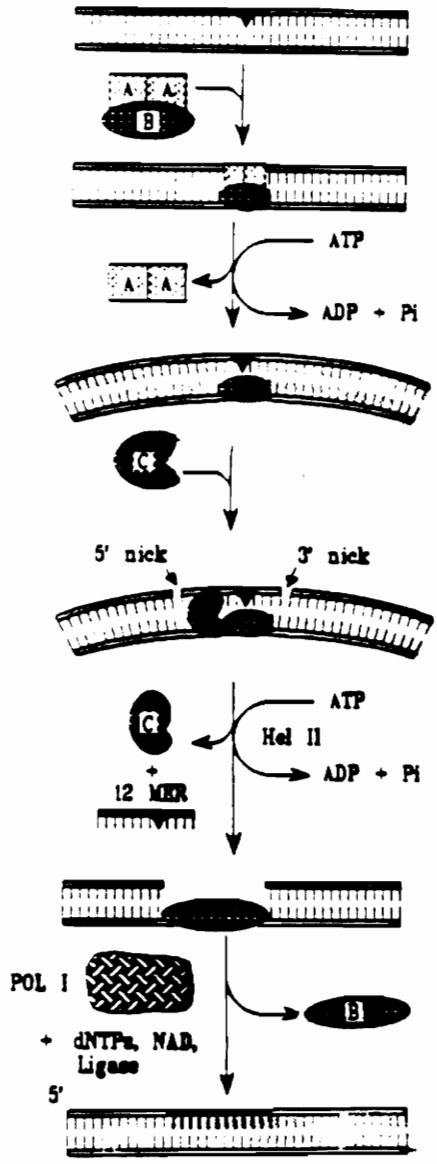


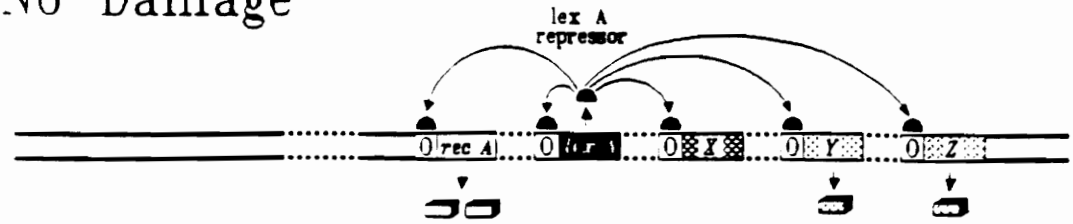
Figure 4. Pathway of nucleotide excision repair in *E. coli*. In the first step, UrvA forms a dimer that complexes to UrvB protein which recognizes and binds to the DNA damage. The UrvA dimer then disassociates and the UrvC protein then binds to the UvrB-DNA damage complex. UrvB and UrvC then nick the DNA 5' and 3' to the damage. DNA helicase II then releases the 12- or 13- mer oligonucleotide and UrvC. UrvB is released when DNA polymerase initiates repair synthesis.

recA regulon controls several of the enzymes involved in nucleotide excision repair including UrvA, UrvB, and UrvC and these enzymes were present only at a basal level in the *recA* mutant. A model of the *lexA-recA* regulon is shown in Figure 5.

Recombinational Repair. Recombinational repair can repair any DNA lesion, provided that an intact copy of the damaged region resides in the same cell. This type of repair can be used when unrepaired damage blocks the progress of DNA replication. When DNA polymerase III encounters DNA lesion, replication stops and restarts at a site beyond the lesion, generating a "daughter-strand gap". By a series of recombinational events, parental DNA from the sister duplex is transferred to fill in the daughter-strand gap. The resulting gaps in the sister strand are then filled by repair synthesis, and the original lesion may be removed by other repair systems, such as base excision repair or nucleotide excision repair before the next cycle of replication. An illustration of the recombinational repair pathway is in Figure 6.

Two types of oxidative damage for which recombination repair is crucial are double-strand breaks and interstrand crosslinks. A double-strand break could be produced by two single-strand breaks, caused by radical attack as described above, occurring on opposite sides of the DNA duplex. Double-strand breaks are not produced in significant amounts by most oxidants, but are among the major products formed by the direct exposure of DNA *in vitro* to some types of ionizing radiation as well as to bleomycin and necarinostatin (Demple and Harrison 1994). Repair of double-strand breaks has a requirement for recombinational repair because the two free ends must be realigned to restore chromosomal integrity (West 1992). RecA is one of the proteins involved in recombinational repair that is under the control of the *lexA-recA* regulon.

No Damage



Synthesis of some gene products is completely repressed, while that of others (e.g., those of *rec A*, *lex A*, genes *Y* and *Z*) is only at a *low* level.

After Damage Occurs

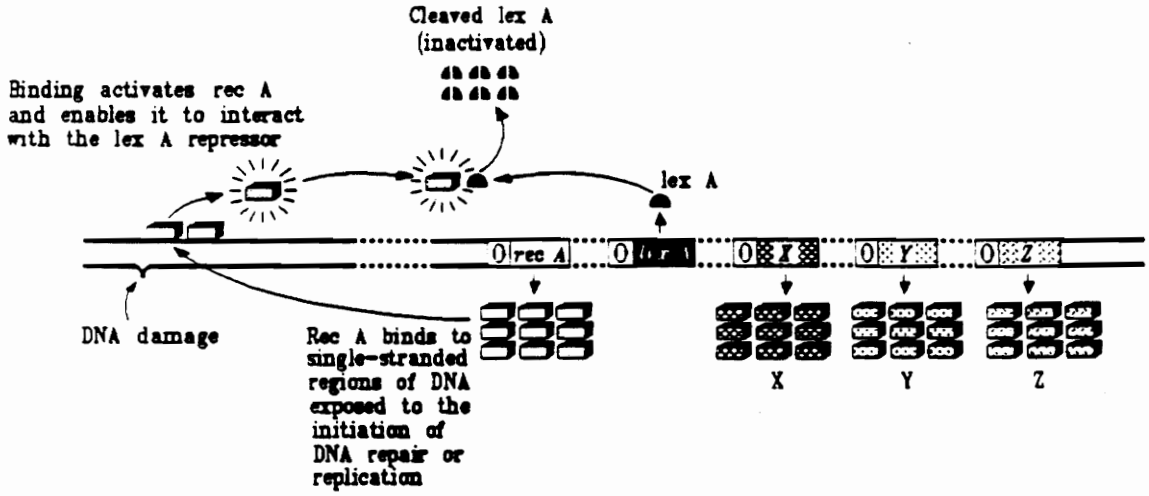


Figure 5. Regulation of *lexA-recA* (SOS) regulon.

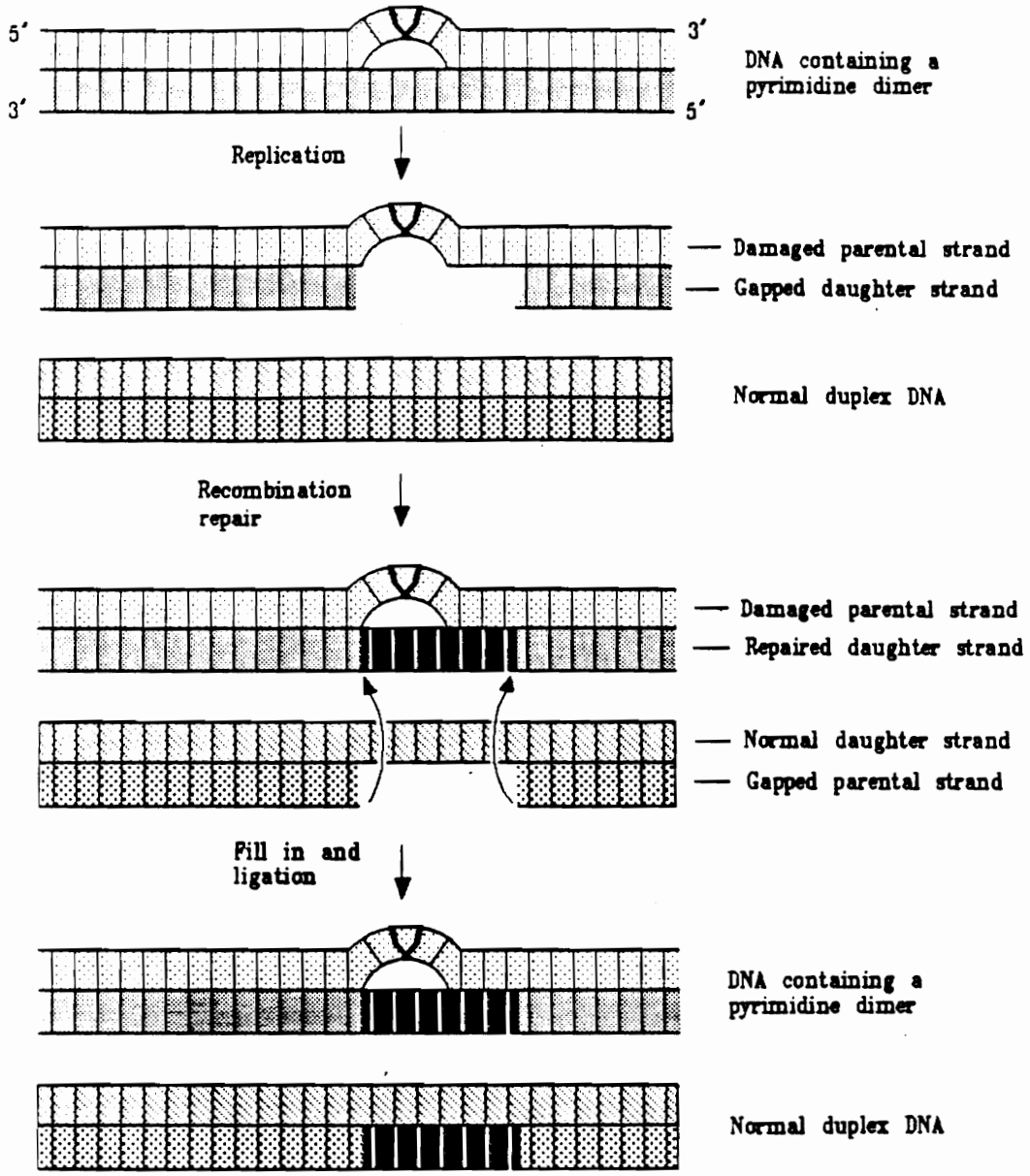


Figure 6. Recombinational repair.

Recombinational repair appears to have a secondary role in the repair of oxidative damage to DNA. While *recA* mutants do show an increased sensitivity to H_2O_2, Hagensee and Moses (1989) found no direct role of the Rec A protein in the repair of H_2O_2. Bruce Demple (personal communication) suggests that *recA* mediated repair is required for double-strand breaks caused by H_2O_2 and to repair daughter-strand gaps that would be created by DNA polymerase III encountering a strand break or other lesion that has not been repaired.

Detection and Measurement of DNA Damage and Repair

There are a number of different methods used to study DNA damage and its repair. Most of these systems are used and applicable for eucaryotic cells only. Many of these methods suffer from one or more of the following limitations: they require the use of radioisotopes, have low sensitivity, are useful only for eucaryotic cells, require special equipment, detect only specific damage, or can be used *in vitro* only.

One of the most common methods to measure single-strand breaks to DNA in bacteria is sedimentation in alkaline sucrose gradients. McGrath and Williams (1966) were the first to use this procedure in which cells are placed on top of an alkaline sucrose gradient, lysed, and then centrifuged. The alkaline component of the gradient denatures the DNA duplex and the single-stranded DNA migrates in the gradient according to size of the resulting fragment. The cells are radiolabeled prior centrifugation and fractions collected from the alkaline gradient after centrifugation are counted to determine the extent of single-strand breakage. The low sensitivity of the assay is attributed to non-specific DNA breaks from shearing forces during centrifugation (Whitaker *et al.* 1991).

Another method used to analyze DNA damage *in vitro* is based on the migration of different conformations of plasmid DNA in an agarose gel. After the DNA is separated in the gel, it is stained

with ethidium bromide and analyzed. If single-strand breaks have occurred, native supercoiled plasmid DNA is converted to a nicked circle. Supercoiled plasmid DNA is converted to linear fragments if a double-strand break occurs. The mobility difference of the conformations of plasmid DNA in an agarose gel is used to estimate the amount of single-strand breaks to the plasmid DNA. This method is useful for analyzing *in vitro* systems, and its main use has been in the determination of which ROS is responsible for DNA strand scission and which scavengers prevent scission (Brawn and Fridovich 1980; Toyokuni and Sagripanti 1992; Devasagayam *et al.* 199; Ozawa *et al.* 1993).

Measurement of DNA synthesis has been used to analyze the repair response of various strains of *E. coli* deficient in DNA repair enzymes (Zhang *et al.* 1992). Immediately after exposure to H_2O_2 or x-rays, the *E. coli* strains were incubated on a medium containing [methyl-^{3}H]thymidine. The uptake of [methyl-^{3}H]thymidine was used to measure the inhibition of DNA synthesis. Survival curves were also performed at the same levels of H_2O_2 and x-ray treatment. A model for repair pathways of oxidative DNA damage was then determined by the different level of DNA synthesis and cell survival in each of the strains. The level of DNA damage in the cell is related to synthesis of DNA because single-strand breaks or other DNA lesions inhibits the progress of replication and the synthesis of DNA. The measurement of DNA synthesis to analyze the repair response of cells is an indirect method to determine the degree of DNA single-strand breakage.

Alkaline elution is employed to measure single-strand breaks in eucaryotic DNA. This method was first described by Kohn and Ewig (1973) and is based on the principle that under alkaline conditions the rate of elution of DNA from lysed cells that are deposited on a membrane filter depends on the length of the single-strands. The conditions for cell lysis and the buffers used to elute the DNA have been shown to cause variations in the sensitivity and results of the assay. This method requires

that the membrane filter "traps" the cells yet has a large enough pore size to elute the DNA after lysis. The pore size required for the DNA elution excludes the used of this method for most bacteria.

Another method used to measure DNA damage is pulsed-field gel electrophoresis (PFGE). In this method, cells are embedding in agarose blocks, lysed and subjected to PFGE. The cells are embedded in agarose to prevent DNA sheering due to manipulation of the cells and DNA. The alteration of field direction and strength in PFGE allows for the separation of large DNA molecules. Stamato and Denko (1990) used γ-irradiated DNA from mammalian cells to show that DNA containing double-strand breaks migrates through the gel, while undamaged DNA remains at the top of the gel.

Other assays are based on an eucaryotic DNA isolation procedure used to produce a "nucleoid" structure to assess DNA damage. In the nucleoid form, DNA is supercoiled and retains some of the higher organization of the nucleus. These methods include nucleoid sedimentation, halo microfluorescence, comet assay, and alkaline unwinding (review in Whitaker *et al.* 1991). In nucleoid sedimentation, the degree of supercoiling modifies the sedimentation of the nucleoid in a sucrose column. In halo microfluorescence, the unwinding of the nucleoid of a single cell is seen as a halo after staining with propidium iodide. In the comet assay, a single cell is embedded in an agarose gel and lysed. The gel is then subjected to electrophoresis and relaxed coils of DNA migrate and form a comet-like shape. In alkaline unwinding, nucleoids are eluted through a hydroxyapatite column and bind differentially to the column according to the amount of unwinding.

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The Use of Alkaline Gel Electrophoresis to Analyze Hydrogen Peroxide-Caused Damage and Repair of DNA in *Escherichia coli*

Abstract

Reactive forms of oxygen such as hydrogen peroxide cause single-strand breaks in DNA. Most of the methods for estimating such breakage and subsequent repair are designed for eucaryotic cells and methods for use with bacteria are needed. Accordingly, a method based on alkaline gel electrophoresis of DNA was developed and tested with isogenic strains of *Escherichia coli* deficient in one or more DNA repair enzymes. Samples from a cell suspension were removed at 2 min intervals following an initial 15 min exposure to 20 mmol l^{-1} hydrogen peroxide. Catalase was added and the cells were embedded in blocks of low-melting point agarose and lysed to liberate their DNA . After alkaline gel electrophoresis, photographs of the gels were taken and the relative lengths of the distributions of DNA fragments were measured with a scanner and computer. The lengths were correlated with survival of the cells exposed to the same concentration of H_2O_2 and with the importance of particular DNA repair enzymes. Alkaline gel electrophoresis appears to be a relatively simple method for analyzing the level of H_2O_2-caused DNA damage and repair in *E. coli*.

INTRODUCTION

Agents such as hydrogen peroxide and X-rays have been shown to induce various types of DNA damage including strand breakage (Hutchinson 1985; Imlay and Linn 1988). Methods for measuring DNA single-strand breaks in intact cells exposed to such agents include alkaline sucrose gradients (MaGrath and Williams 1966), alkaline elution (Kohn and Ewig 1973), and alkaline unwinding (Ahnström and Edvardsson 1974). The alkaline sucrose gradient is sometimes considered the standard method for measuring single-strand breaks but it is relatively insensitive and requires radiolabeling of the cells and several laborious steps. The alkaline elution and alkaline unwinding methods depend on the structure of the eucaryotic nucleus or on the relatively large size of eucaryotic cells and are not readily applicable to bacteria.

This article reports the use of alkaline gel electrophoresis for visualizing and measuring the extent of DNA single-strand breaks and subsequent repair in intact cells of *Escherichia coli* exposed to H_2O_2. Alkaline gel electrophoresis has been used to analyze single-strand breaks in human cells (Kovacs and Langemann 1990) and to investigate the effect of gamma rays on DNA in vitro (Chen and Sutherland 1989). The advantages of alkaline gel electrophoresis are that it is relatively easy, economical, rapid and does not involve the use of radioisotopes. We compared the results from a wild type *E. coli* strain to those from isogenic mutants that were deficient in specific DNA repair enzymes.

MATERIALS AND METHODS

Bacterial strains. The strains of *Escherichia coli* used were isogenic and are listed in Table 1. Working stocks were maintained aerobically in Luria-Bertani (LB) broth (10 g l^{-1} tryptone, 10 g l^{-1} NaCl, 5 g l^{-1} yeast extract) at 37°C with daily serial transfer. Fresh working stock cultures were prepared every two weeks from one of the multiple frozen aliquots of an initial culture.

Survival of H_2O_2 treated cells. Cells were grown aerobically in 25 ml of LB broth on an orbital shaker (125 rev. min^{-1}) to an $O.D._{620}$ of 0.5 (2 x 10^8 cfu ml^{-1}) as measured with a 16-mm round cuvet and a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, NY). The cells were harvested at 10,000 x g for 10 min at 4°C, washed twice in cold phosphate-buffered saline (PBS; 140 mmol l^{-1} NaCl, 10 mmol l^{-1} Na_2HPO_4, 10 $mmol^{-1}$ KH_2PO_4, pH 7.2), suspended in 25 ml of PBS and warmed to 37°C. Hydrogen peroxide was added from a spectrophotometrically analyzed stock solution to a final concentration of 20 mmol l^{-1}. Samples were removed periodically from the H_2O_2-treated culture, diluted in PBS containing bovine catalase (800 units ml^{-1}, Sigma Chemical Co., St. Louis, MO) to destroy the H_2O_2 and spread onto LB agar plates. Colonies were counted after overnight incubation at 37°C.

Determination of DNA Damage and Repair. A 25 ml culture was grown to $O.D._{620}$ of 0.5 and the cells were harvested and suspended in 25 ml of PBS as described above. A 1 ml sample was centrifuged for 30 sec at 15 000 x g, washed in Tris-EDTA (TE) buffer (50 mmol l^{-1} TRIS-HCl, 5 mmol l^{-1} EDTA, pH 8.0) at 4°C, suspended in 10 µl of TE and placed on ice. This sample served as the pre-H_2O_2 exposure control. Hydrogen peroxide was then added to the main cell suspension to a

Table 1. *Escherichia coli* strains used in this study

Strain	Relevant genotype*	Source
AB1157	*xth+ pol+ rec+*	Mary Berlyn, *E. coli* Genetic Stock Center, Yale University, New Haven, CT
BW9091	*xthA1*	Mary Berlyn
JC2924	*recA56*	Mary Berlyn
SK2237†	*polA1*	Sidney Kushner, University of Georgia, Athens
AB3027‡	*polA20 - xthA14*	Mary Berlyn

* All strains are isogenic with the following traits: F,- *thr-1*, *ara-14*, *leuB6*, Δ(*gpt-proA*)62, *lacY*1, *tsx-33*, qsr', *glnV44*, galK2, λ-, rac-, *hisG4*, *rfbD1*, *mgl-51*, *rpsL31*, *kdgK51*, *xylA5*, *mtl-1*, *argE3*, *thi-1*

† Strain SK2237 also contains the following markers: *lacMS268*, *lacBK1*, *ilvA*

‡ Strain AB3027 also contains the following marker: *rha-6*

final concentration of 20 mmol l^{-1}. After 15 min a 1 ml sample was centrifuged and treated as described above; at the same time, bovine liver catalase was added to the main cell suspension to a final concentration of 20,000 U ml^{-1}. A 1 ml sample was removed at 1 min after the addition of catalase, centrifuged and treated as described above. Additional samples were removed every two min for 15 min.

Samples were analyzed for single-strand DNA breaks by alkaline gel electrophoresis. Fifty microliters of 1% low melting point agarose (Fisher Scientific Co., Silver Spring, MD) at 37°C was added to each 10 µl sample. The agarose and cells were mixed thoroughly and 60 µl blocks were made by pipetting the mixture onto Parafilm M®. After solidification, the blocks were incubated at 55°C overnight in a lysing solution consisting of 0.25 mmol l^{-1} EDTA, 0.5% sodium-N-lauroyl-sarcosinate [wt vol^{-1}] and 0.5 mg ml^{-1} proteinase K (Sigma). The blocks were washed three times (10 min each) in cold TE and placed onto the teeth of the comb of a Model 1214 gel electrophoresis apparatus (Fotodyne Corporation, New Berlin, WI). Removal of excess buffer with filter paper facilitated adherence of the blocks to the comb. The gel was cast with a 0.76% solution of high melting DNA grade agarose (Fisher) containing 0.03 mmol l^{-1} NaOH and 0.01 mmol l^{-1} EDTA. Kilobase ladder DNA (Bethesda Research Laboratories, Bethesda MD) in concentration of 0.8 µg $lane^{-1}$ was used as a size standard. The gel was then subjected to electrophoresis at 30 V for 6 h at 22°C. The Model 1214 apparatus allowed electrophoresis of two 6.5 cm by 12 cm gels simultaneously. Each gel was neutralized by soaking in a 500 ml solution containing 0.03 mol l^{-1} NaCl and 0.05 mol l^{-1} Tris-HCl at pH 6.0 for 1 h. The gel was stained in a solution containing 0.5 µg ml^{-1} ethidium bromide for 45 min and destained in double-distilled water for 10 min. Gels were photographed with a Model UV300 UV transilluminator (Fotodyne), a P-MP4 Land Camera (Polaroid Corporation, Cambridge, MA) and 667

film (Polaroid). The exposure time was 4 sec and the camera lens positioned 31 cm and film 53.5 cm from the gel respectively. Each photograph was scanned with a Color OneScanner scanner (Apple Computer Inc., Cupertino CA) using Ofoto version 2.0.2 software (Light Source Computer Images Inc., Larkspur CA). The image was imported into NIH Image version 1.55 (National Institutes of Health, Bethesda MD) and the "magic wand" function was used to outline a border around each DNA distribution. The length of the distribution was then measured by using "measure length" from the "options" menu of NIH Image. The relative length of each distribution was expressed in terms of the measured length divided by the length of the 1.0 kb band of the DNA standard.

RESULTS

Survival of H_2O_2 treated cells. Cell survival after exposure to a 20 mmol l^{-1} dose of H_2O_2 is shown in Figure 1. All the mutant strains were more readily killed by the H_2O_2 treatment than the wild type cells. Of the mutant strains, *recA* was the least sensitive followed by *xthA*, *polA*, and *xthA-polA*.

Estimation of Damage and Repair. Examples of a typical alkaline gel of each strain are shown in Figures 2 to 6. The pre-H_2O_2 exposure control cells (0 min), show no DNA migration from the blocks, indicating negligible amount of DNA single-strand breakage. The wild type cells showed increase in length of the DNA distribution (15 min) indicating a moderate amount of DNA single-strand breakage after 15 min exposure to 20 mmol l^{-1} H_2O_2. After the addition of bovine catalase, much of this damage was repaired, indicated by the decrease in lengths of the DNA distribution during the next 15 min (16-30 min). The amount of damage was quantified by measuring the relative lengths of the DNA distributions (Figure 7). The *recA* mutant showed DNA distributions having only slightly greater relative lengths than that of the wild type cells. The *xthA* mutant showed an increase in the relative lengths after the 15 min H_2O_2 exposure, as compared with the wild type strain. The *polA* mutant and the *polA-xthA* double mutant showed the greatest levels of increase in the relative lengths of the DNA distributions.

Comparison of Cell Survival and Relative Length. Cell survival was plotted against the relative length of the DNA distribution of each strain after 15 min exposure to 20 mmol l^{-1} H_2O_2 (Figure 8). The values of cell survival of the *xthA*, *polA*, and *xthA-polA* strains at 15 min were extrapolated using linear regression from the data in Figure 1.

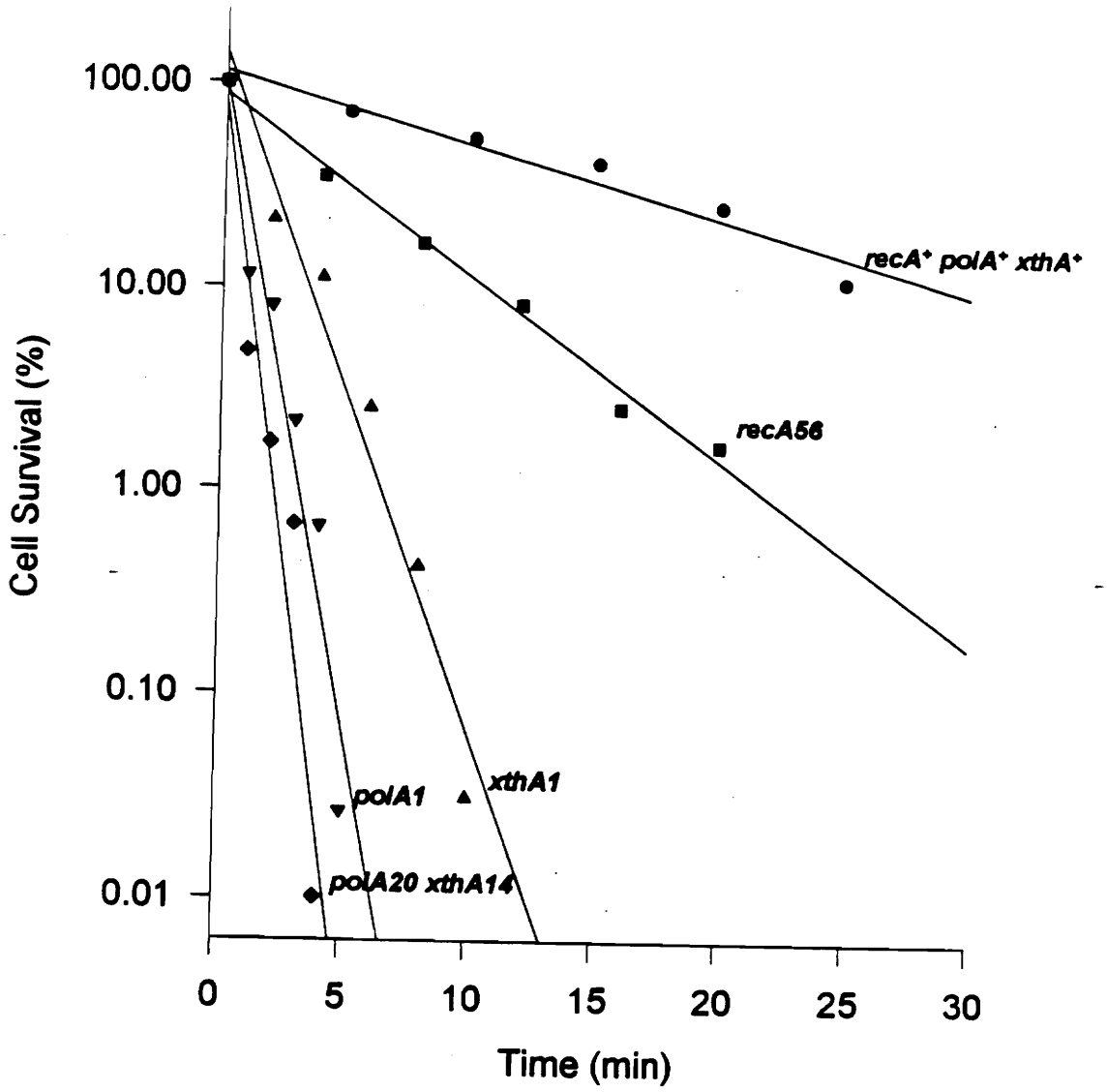

Figure 1. Cell survival after exposure to 20 mmol l^{-1} H_2O_2. Each point represents the average of three experiments done in triplicate.

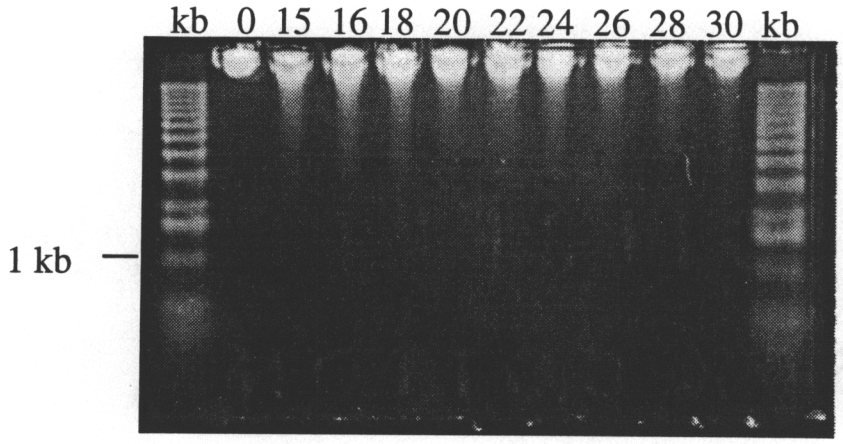

Figure 2. Alkaline gel of *E. coli* AB1157 (*xthA*$^+$ *recA*$^+$ *polA*$^+$). kb is kilobase ladder. Numbers are minutes after the addition of 20 mmol l^{-1} H_2O_2. Catalase was added at 15 min.

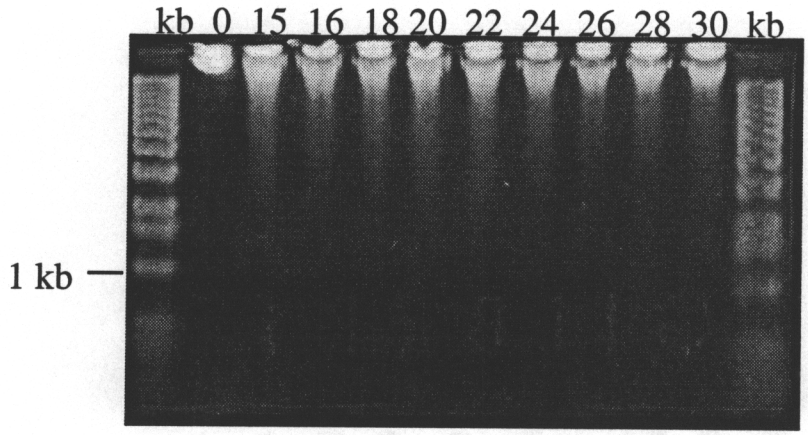

Figure 3. Alkaline gel of *E. coli* JC2924 (*recA56*). Lanes are as described in Figure 2.

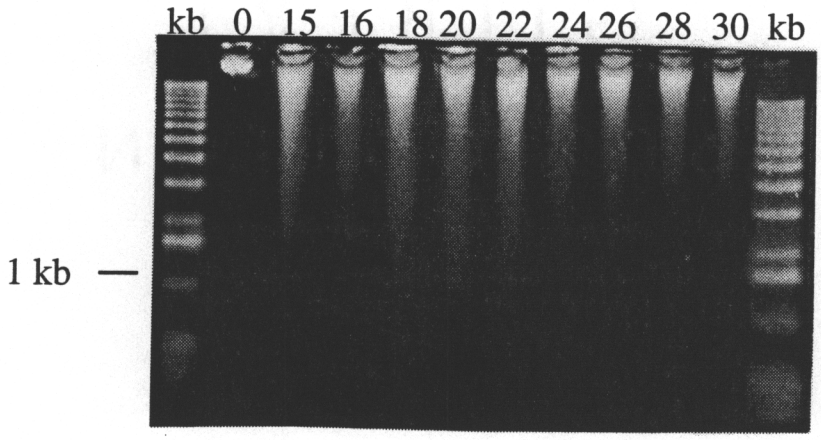

Figure 4. Alkaline gel of *E. coli BW9091 (xthA)*.
Lanes are as described in Figure 2.

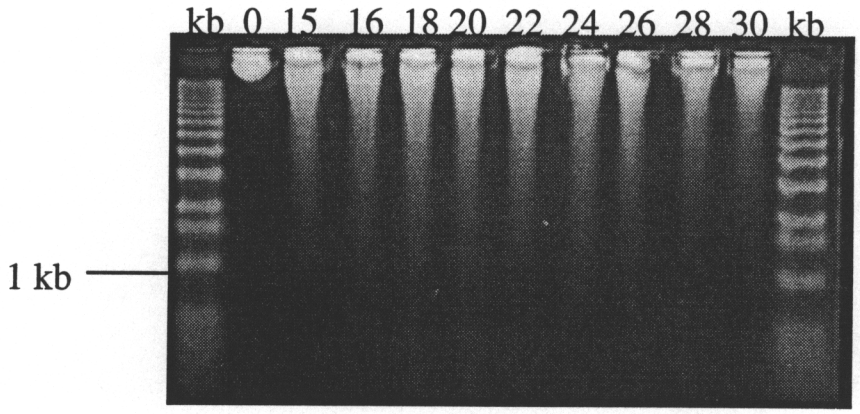

Figure 5. Alkaline gel of *E. coli* SK2237 (*polA*).
Lanes are as described in Figure 2.

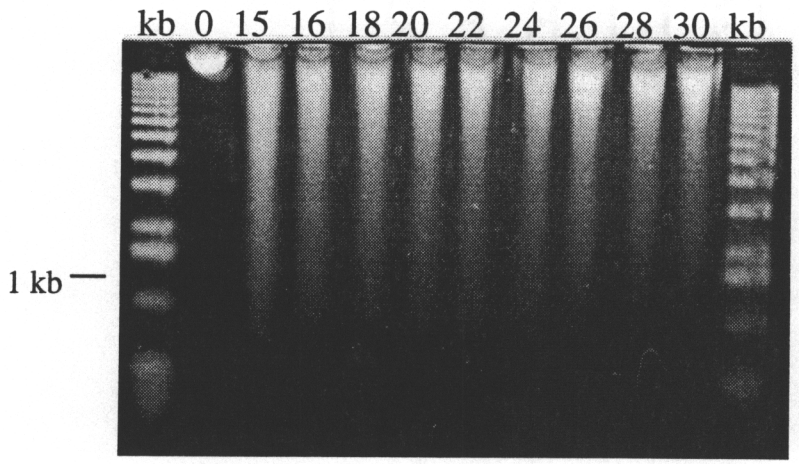

Figure 6. Alkaline gel of *E. coli* AB3027 (*polA-xthA*). Lanes are as described in Figure 2.

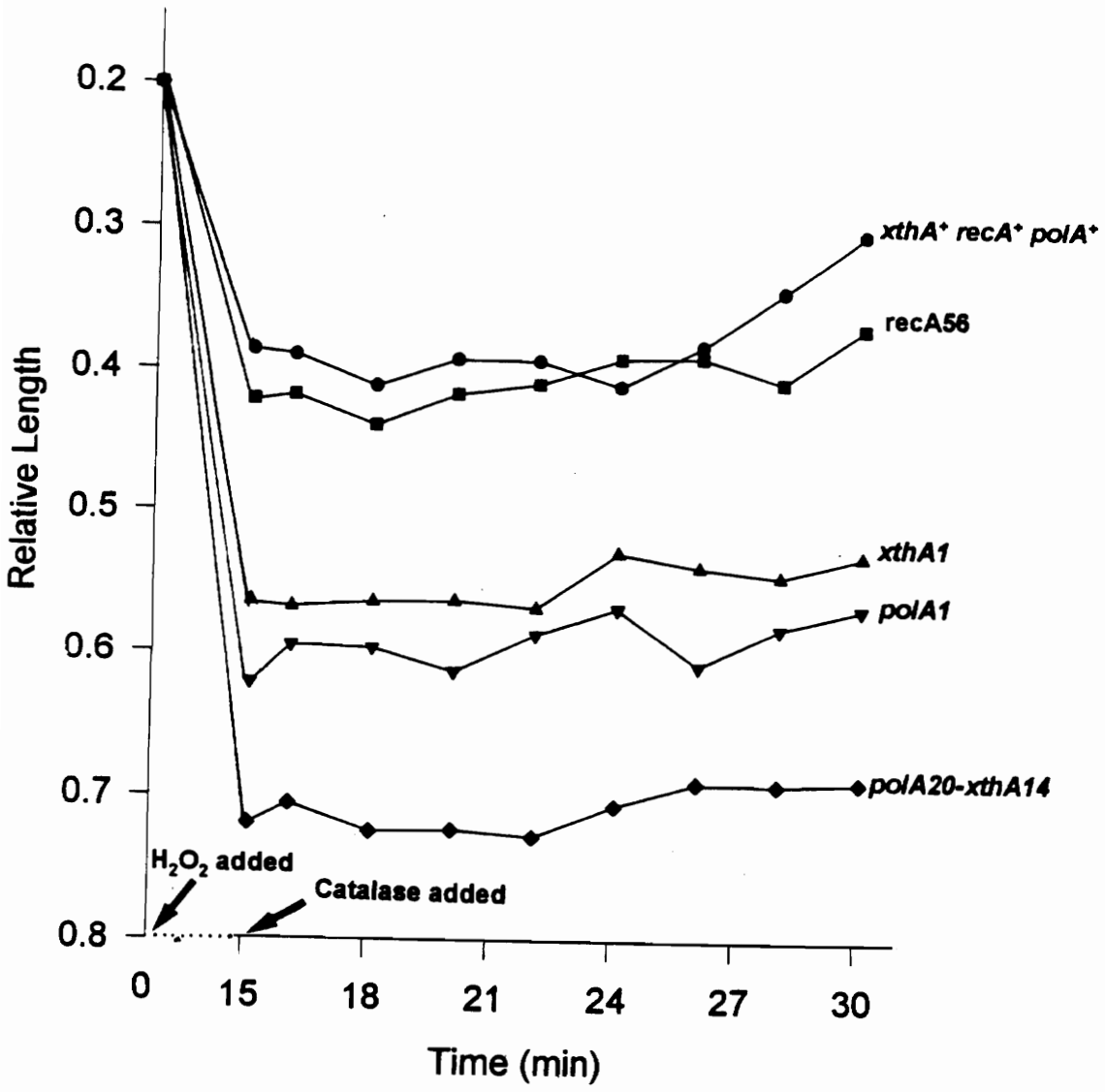

Figure 7. Relative length of DNA distributions in alkaline gels after the addition of 20 mmol l^{-1} H_2O_2. Catalase was added at 15 min. Data represent a typical gel of each strain.

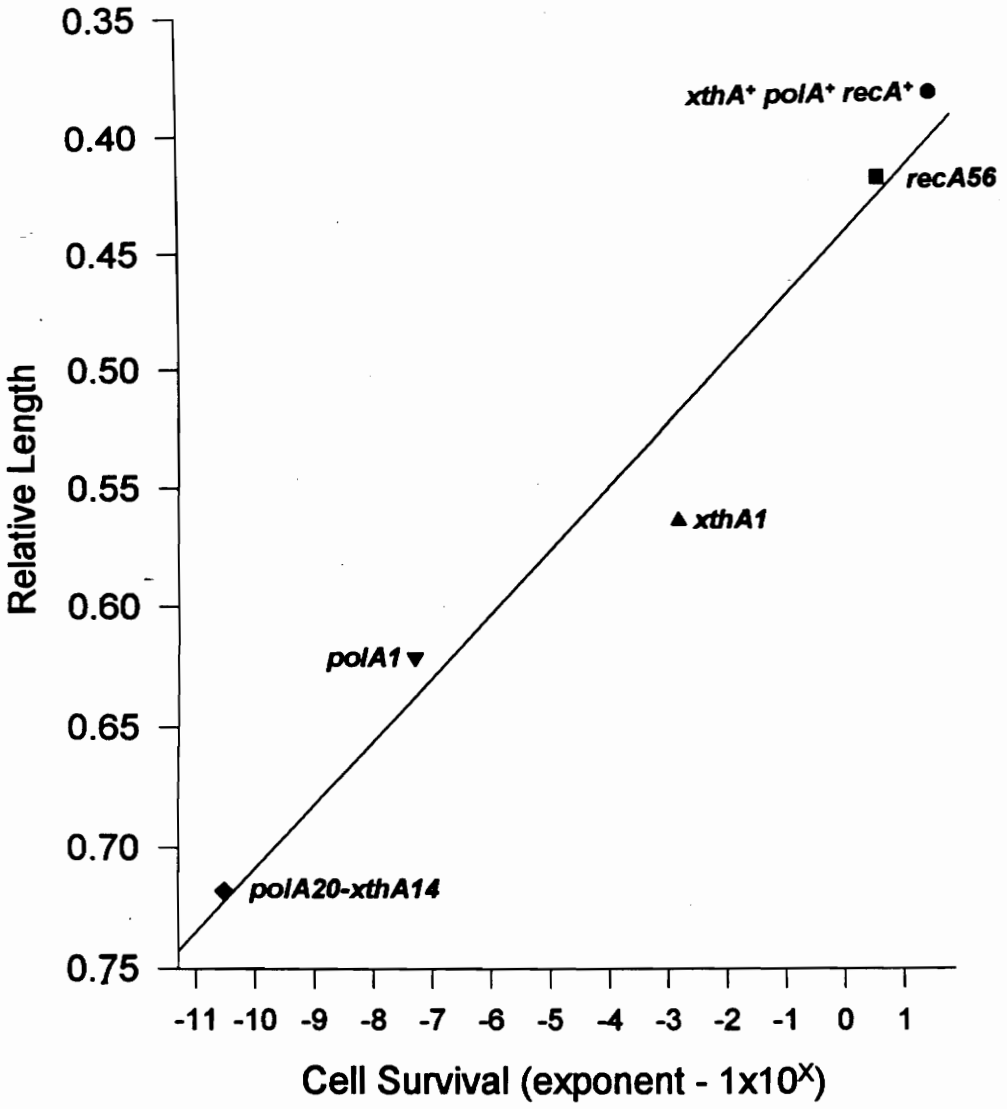

Figure 8. Relative length of DNA distributions verses cell survival of each strain after 15 min exposure to 20 mmol l^{-1} H_2O_2

DISCUSSION

The purpose of this study was to develop a relatively simple and economical method based on alkaline gel electrophoresis to analyze the single-strand breakage of DNA and subsequent repair in whole bacterial cells exposed to H_2O_2. Hydrogen peroxide is known to cause single-strand breaks to DNA. When the double-stranded DNA is subsequently denatured in an alkaline agarose gel, various sizes of DNA single-stranded fragments migrate differently through the gel when subjected to electrophoresis, generating a distribution whose length depends on the extent of DNA single-strand breakage. The gels can be photographed and lengths of the DNA distributions measured with a computer and scanner. To minimize DNA shearing due to mechanical manipulation of high molecular weight DNA, the bacteria were embedded in low melting point agar prior to lysis and electrophoresis. In testing the method, *Escherichia coli* strains were used because of the detailed knowledge of DNA repair pathways in this organism and because isogenic strains of *E. coli* were available that were deficient in specific repair enzymes.

Parameters affecting the methodology. When performing this method, cells should always be grown to the same density prior to harvesting and treatment with H_2O_2. Changes in cell density caused variation in the number of cells and therefore the amount of DNA in each agarose block. The use of fewer then 2×10^8 cfu ml^{-1} prior to harvesting resulted in reduction in intensity and length of the DNA distribution in the gels.

The use of PBS as suspending medium for the cells prior to H_2O_2 treatment is also important. Although similar results were obtained when cells were suspended in LB or PBS for three of the strains, the DNA distribution lengths of strains AB1157 and JC2924 were greatly reduced in LB. The possibility that LB components decreased the H_2O_2 concentration was eliminated. When H_2O_2 was

added to sterile blanks of LB, PBS and water, the H_2O_2 concentrations were the same for all three solution as measured by a colorimetric assay (PerXOquant, Pierce Chemical Co., Rockford IL)

Low-melting point agarose for embedding the cells was preferable to high-melting point agarose. The former could be mixed with the cells and pipetted to make blocks before solidification occurred, whereas high-melting point agarose solidified too quickly for this purpose.

Electrophoresis voltages less than 0.7 v cm^{-1} at 22 C resulted in longer run times although similar DNA distributions eventually resulted. Voltages greater than 0.7 v cm^{-1} caused "smiles" to occur in the kilobase ladder as well as "bends" in the DNA distributions. Gels electrophoresed at 4°C showed a substantial decrease in the length of the distributions of all the strains tested. This might be attributable to less denaturation of the DNA duplex at 4°C.

The concentration of agarose used in the gels affected the quality of the DNA profiles. The agarose concentration was varied between 0.6% and 1.0%. At an agarose concentration of 0.76%, the gels contained longer distributions than gels prepared with 1.0% agarose and they were easier to handle than gels prepared with 0.6% agarose.

Variation in the concentration of neutralizing buffer seemed to have minimal effects, and gels could even be neutralized overnight in distilled water.

The use of constant ethidium bromide concentration and staining time was important because an increase or decrease in these parameters caused a corresponding increase or decrease in the length of the DNA distribution. Maintaining a constant camera height when photographing the gel and exposure time of the film was also important. Consequently, the camera height was always measured prior to photographing the gel and the exposure time was precisely controlled by an electric timer on the transilluminator.

Effect of H_2O_2 on *E. coli*. The survival curves of the H_2O_2-treated cells showed that all four mutant strains, *recA*, *xthA*, *polA*, and *xthA-polA*, had increased sensitivity as compared to the wild type

cells (Figure 1). The analysis of the DNA distributions by alkaline gel electrophoresis after 15 min H_2O_2 exposure showed increased initial relative lengths for all the strains as compared to the control cells. These increases indicate an increased degree of DNA single-strand breakage. Isogenic daughter strains of the wild type (AB1157) were used in this study to minimize the possibility that the differences in relative lengths of the DNA distributions could be due to some other factor other than the mutations in DNA repair enzymes. When the relative lengths of the distributions were plotted against the survival after 15 min exposure to 20 mmol l^{-1} H_2O_2 for each strain (Figure 8) a linear relationship was obtained, suggesting that cell survival is related to the relative length values.

All samples were taken within 30 min of the addition of H_2O_2 to minimize the possibility of analyzing newly synthesized DNA. In *E. coli*, in the presence of a DNA damaging agent, DNA synthesis is transiently inhibited and recovers in approximately 30-45 min (Livneh *et al.* 1993; Echols and Goodman 1990).

The wild type cells were the least sensitive to the H_2O_2 (Figure 1) and showed the smallest increase of relative length of the DNA distributions after the 15 min H_2O_2 exposure (Figure 8). After the addition of catalase, the relative lengths decreased and approached the values of the control cells. This suggests that the wild type cells were the most efficient in the repair of single-strand DNA breaks.

Of the mutant stains, the *recA* strain showed the least sensitivity to H_2O_2 (Figure 1). This was in accord with results obtained by Ananthaswamy and Eisestrak (1977), Demple *et al.* (1983) and Hagensee and Moses (1989). The findings that the initial relative lengths of the DNA distributions were only slightly greater then that of the wild type cells and of the subsequent repair of these single-strand breaks, indicated by the reduction in relative length after the addition of catalase (Figure 7) indicate that recA-dependent repair is not a primary pathway in the repair of single-strand breaks caused by hydrogen peroxide in *E. coli*. This is consistent with the results of DNA repair studies by Hagensee and Moses (1989) and Demple and Harrison (1994).

The *xthA* mutant cells were extremely sensitive to H_2O_2 (Figure 1). In *E. coli*, exonuclease III has the activity of a class II endonuclease, which cleaves 5' to AP sites and has associated 3' phosphatase and 3' phosphodiesterase activities (Doetsch and Cunningham 1990; Demple et al. 1986). Strand breaks caused by H_2O_2 usually contain 3'-phosphorylated termini are not substrates for *E. coli* DNA polymerase I and are considered blocked (Demple et al. 1985). The associated 3'-phosphatase and 3'-phosphodiesterase activities of exonuclease III remove blocked 3'-termini and generate 3'-hydroxy termini required to initiate repair synthesis by DNA polymerase I (Weiss 1981, Demple et al. 1986; Doetsch and Cunningham 1990). The *xthA* mutants showed at large increase of initial relative length of their DNA distributions compared to the wild-type cells. The lengths decreased slightly after the addition of catalase, indicating that some repair did occur (Figure 4). This suggests that a large portion of single-strand DNA breaks caused by H_2O_2 do contain blocked 3' termini that require processing to generate 3' OH termini for resynthesis by DNA polymerases. Some of the strand breaks in the *xthA* mutants could be repair by endonuclease IV. Endonuclease IV is also a class II AP endonuclease and has associated activities in common with exonuclease III, but exonuclease III accounts for approximately 99% of 3' phosphatase activity in *E. coli* (Demple *et al.* 1983).

The *polA* mutants also showed extreme sensitivity to the H_2O_2 (Figure 1). These cells which lacked functional DNA polymerase I also gave long DNA distributions and only a slight amount DNA repair occurred (Figure 5). DNA polymerase I is responsible for initiating repair synthesis in DNA repair. Hagensee and Moses (1989) initially reported that DNA polymerase I was required in the repair of any oxidative damage but Zhang *et al.* (1992) reported that some oxidative DNA damage could be repaired by DNA polymerase III. Our results suggest that some DNA single-strand breaks can be repaired in a pathway independent of DNA polymerase I.

The *polA-xthA* strain showed the highest initial relative DNA distribution length after H_2O_2 treatment and only a slight reduction of these lengths after the addition of catalase (Figure 6).

Deficiency in both exonuclease III and DNA polymerase I activities renders these cells extremely sensitive to H_2O_2. Some slight repair of single-strand breaks did occur however, as shown by the slight decrease in relative length of the DNA distributions after the addition of catalase to the cells. The ability of this mutant to repair some of the single-strand breaks suggests that repair might occur by a pathway independent of exonuclease III-DNA polymerase I.

In conclusion, a method was developed that allows analysis of DNA single-strand breaks and subsequent repair in intact cells *E. coli* exposed to H_2O_2, as measured by the lengths of DNA distributions in alkaline gels. The relative lengths of the DNA distributions were correlated with the degree of survival of the H_2O_2 treated cells. The results were also consistent with previous studies of *E. coli* regarding the relative importance of particular enzymes in DNA repair. Although the alkaline electrophoresis method was applied to *E. coli*, the method probably could be applied to other bacteria as well.

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Appendix A

Chromosomal Markers of the *Escherichia coli* Strains Used in this Study

Strain: AB1157

Chromosomal Markers: *thr-1*, *ara-14*, *leuB6*, Δ(*gpt-proA*)62, *lacY*1, *tsx-33*, qsr^{-}, *glnV44*(AS), galK2, λ-, rac-, *hisG4*(Oc), *rfbD1*, *mgl-51*, *rpsL31*, *kdgK51*, *xylA5*, *mtl-1*, *argE3*(Oc), *thi-1*

Strain: JC2924

Chromosomal Markers: *thr-1*, *ara-14*, *leuB6*, Δ(*gpt-proA*)62, *lacY*1, *tsx-33*, qsr^{-}, *glnV44*(AS), galK2, λ-, rac-, *hisG4*(Oc), *rfbD1*, *mgl-51*, *rpsL31*, *kdgK51*, *xylA5*, *mtl-1*, *argE3*(Oc), *thi-1*, ***recA56***

Strain: BW9091

Chromosomal Markers: *thr-1*, *ara-14*, *leuB6*, Δ(*gpt-proA*)62, *lacY*1, *tsx-33*, qsr^{-}, *glnV44*(AS), galK2, λ-, rac-, *hisG4*(Oc), *rfbD1*, *mgl-51*, *rpsL31*, *kdgK51*, *xylA5*, *mtl-1*, *argE3*(Oc), *thi-1*, ***xthA1***

Strain: SK2237

Chromosomal Markers: *thr-1*, *ara-14*, *leuB6*, Δ(*gpt-proA*)62, *lacY*1, *tsx-33*, qsr^{-}, *glnV44*(AS), galK2, λ-, rac-, *hisG4*(Oc), *rfbD1*, *mgl-51*, *rpsL31*, *kdgK51*, *xylA5*, *mtl-1*, *argE3*(Oc), *thi-1*, ***polA1***, ***lacMS268***, ***lacBK1***, ***ilvA***

Strain: AB3027

Chromosomal Markers:

thr-1, *ara-14*, *leuB6*, Δ(*gpt-proA*)62, *lacY*1, *tsx-33*, qsr^{-}, *glnV44*(AS), galK2, λ-, rac-, *hisG4*(Oc), *rfbD1*, *mgl-51*, *rpsL31*, *kdgK51*, *xylA5*, *mtl-1*, *argE3*(Oc), *thi-1*, ***xthA14***, ***polA20***, ***rha-6***

Comments: Boldface type indicates markers that differ from AB1157. AS are amber (UGA) suppressors and Oc are ochre (UAA) mutations.

Curriculum Vitae

Name: Ross E. Zirkle

Birth: July 15, 1971 - York, Pennsylvania

Home: 7323 Gumwood Lane
Raleigh NC, 27615
Phone: 919/878-8120

Education:

1993-Present M.S. in Biology in progress, Virginia Tech, Blacksburg, VA.

1993 B.S. in Biochemistry, Virginia Tech, Blacksburg, VA.

Thesis Title: The Use Of Alkaline Gel Electrophoresis to Analyze Hydrogen Peroxide-Caused DNA and Repair in *Escherichia coli*

Major Professor: Dr. Noel R. Krieg, Alumni Distinguished Professor

Professional Experience:

1993-1995

Graduate Teaching Assistant: Biology Department, Virginia Tech, Blacksburg, VA. Pathogenic Bacteriology Laboratory, General Microbiology Laboratory, Principles of Biology Laboratory, and General Biology Laboratory

1991-1993

Laboratory Technician: Chemistry Department, Virginia Tech, Blacksburg, VA. Laboratory of James McGrath, Ph.D.

Grants:

1994

Sigma-Xi Grant-in-Aid of Research - "DNA Damage and Repair in Microaerophiles" received on July 9. ($400 plus matching amount from Dept.)

Presentations:

1994

"Repair to Oxidative Damage of DNA," Microbiology and Immunology Seminar Series. Biology Department, Virginia Tech, Blacksburg, VA. 26 October.

Published Abstracts:

1995

Zirkle, R. E. and Krieg N. R. "Visualization of DNA Damage and Repair in *Escherichia coli* by Alkaline Agarose Gel Electrophoresis". Abstracts of the 95th General Meeting of the American Society for Microbiology. Washington DC. Page 560. Poster K-139 24 May.

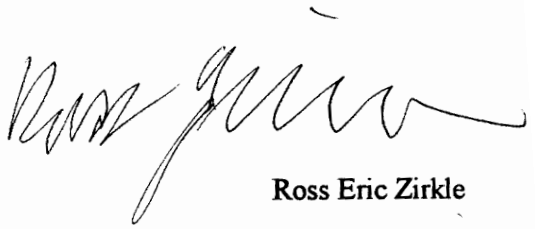
Manuscripts in Preparation:

1995

"The Use of Alkaline Gel Electrophoresis to Analyze Hydrogen Peroxide-Caused DNA Damage and Repair in *Escherichia coil*". To be submitted to the Journal of Applied Bacteriology.

Professional Memberships:

American Society of Microbiology



Ross Eric Zirkle