

CHARACTERIZATION OF DNA-REPAIR POTENTIAL IN
DEEP SUBSURFACE BACTERIA CHALLENGED BY UV LIGHT,
HYDROGEN PEROXIDE, AND GAMMA RADIATION

by

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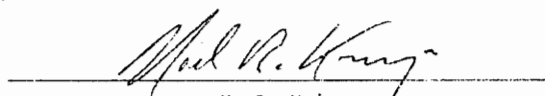
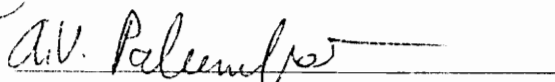
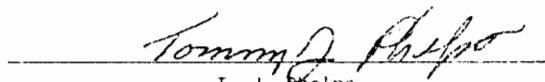
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(ABSTRACT)

Subsurface bacterial isolates obtained through the DOE Subsurface Science Program were tested for resistance to UV light, gamma radiation and H_2O_2 . Some deep subsurface bacteria were resistant to UV light, demonstrating $\geq 1.0\%$ survival at fluences which resulted in a 0.0001% survival level of E. coli B. The percentage of UV resistant aerobic subsurface bacteria and surface soil bacteria were similar; 30.8% and 25.8% respectively. All of the microaerophilic subsurface isolates were UV sensitive as defined in this work; however, subsurface isolates demonstrated UV resistance levels similar to reference bacterial strains of the same Gram reaction. These results were not in agreement with the hypothesis that the resistance of an organism to UV is correlated with the amount of solar radiation in its natural habitat. Evidence for photoreactivation and the presence of an SOS-like mechanism was also detected in subsurface bacteria. The presence of UV resistance and photoreactivation in subsurface bacteria that have been shielded from solar radiation for millions of years may point to a limited rate of evolution in

the deep subsurface environment.

In subsurface bacteria, there was a relatedness between UV resistance and resistance to gamma radiation and H_2O_2 . UV-resistant aerobic subsurface isolates were also gamma and H_2O_2 -resistant compared to the microaerophilic isolates tested. Due to the similarities of bacterial responses to UV, H_2O_2 , and gamma radiation, either UV or H_2O_2 may be utilized to model the effects of ionizing radiation on bacterial cultures used for the bioremediation of organic and radioactive waste-containing environments.

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1.0. INTRODUCTION

The reproduction and evolution of all life depends on the maintenance of a cell's genetic information. This genetic material is challenged by a myriad of potentially lethal agents that may damage the integrity of the DNA molecule. To oppose these challenges, cells have developed enzymatic pathways to remove and repair DNA damage. A number of DNA repair mechanisms have been characterized in Escherichia coli (12,47,54,56,58) and other bacteria (1,11,32,33,34,43) including excision repair, photoreactivation, post-replicative repair and SOS repair.

The ability to repair DNA damage is essentially universal in naturally occurring bacteria (32), though there is variability in the repair efficiencies between different bacterial species and strains (31,33). Not all bacteria possess a full complement of DNA repair mechanisms. For example, Deinococcus radiodurans and Haemophilus influenzae lack photoreactivation (19).

Recent studies have uncovered a diverse microbial population predominated by bacteria in the deep subsurface environment (3,4,5,9,16,30,39,44). Molecular (25) and biochemical (3,4) analyses suggest that these microorganisms have evolved in place for millions of years with limited contact from surface microorganisms. Current investigations of deep subsurface bacteria have focused on the isolation of

microorganisms that can degrade or transform toxic compounds from polluted groundwater and terrestrial sites (6,16,21,52,53). The suitability of bacteria for the bioremediation of organic and mixed wastes in the subsurface environment will depend in part on their capacity to tolerate DNA damage. Microorganisms must be able to carry out their metabolic activities under the stressful conditions encountered in a polluted environment.

The goals of this study were to evaluate the DNA-repair potential of subsurface bacteria relative to other known bacterial isolates and to develop practical protocols for testing bacterial tolerance to ionizing radiation. Bacteria were exposed to three treatments: UV light, H_2O_2 and gamma radiation. Survival curves were examined to gauge the performance of these bacteria in a mixed waste environment. In addition, the treatments were compared to determine the feasibility of utilizing UV and H_2O_2 to model bacterial resistance to gamma radiation.

2.0. LITERATURE REVIEW

2.1. DNA DAMAGE

2.1.1. Ultraviolet radiation damage

The ultraviolet (UV) region of the electromagnetic spectrum is located between X-rays and visible light, and includes the wavelengths of 100 - 380 nm. Although UV has a higher energy level than the visible range it does not directly possess molecular ionizing capabilities. The UV spectrum is subdivided into 4 areas: UV-A 380-315 nm; UV-B 315-280 nm; UV-C 280-200 nm and vacuum UV which is composed of wavelengths below 200 nm which are absorbed by air (31). The most effective bactericidal activity occurs within the UV-C region. Monochromatic sources of 254 nm or 265 nm light are most often utilized for laboratory experiments.

The effects of UV light on bacterial survival have been known for over a century. One of the first accounts of the bactericidal nature of UV was published by Downes and Blunt in 1877 (15). In the last 60 years, investigators have provided insight into the nature of UV-induced DNA lesions and the mechanisms utilized by bacteria to repair and therefore tolerate the damage.

An initial problem was determining the UV inactivation target. Early efforts concentrated on proteins and nucleic acids. Both have absorption peaks in the UV region: proteins around 280 nm and nucleic acids around 260 nm (45). By the

middle of this century, it was generally agreed that nucleic acids were in fact the major target of UV. The UV irradiation of DNA can produce many types of photoproducts. However it is thought that cyclobutane pyrimidine dimers, especially those of thymine, are the major lesions responsible for the lethal and mutagenic effects of UV (19). The 5,6-double bond of thymine has a strong absorption in the UV-C region. When a photon is absorbed by a monomeric thymine molecule, the double bond is split and a cyclobutane ring is formed with the adjacent thymine. There are six isomers of the thymine dimer that can exist in solution; however, due to the structure of the alpha helix, only the cis-syn form of the dimer is possible in dsDNA (45).

The induction of a thymine dimer is not inherently lethal to a cell. Only when the irradiated DNA undergoes replication does the presence of a dimer become potentially lethal. Normally, there are no nucleotide bases which can be added across the dimer so a gap is left in the newly synthesized daughter strand of DNA (45). These single strand breaks, resulting from the replication of irradiated DNA, are lethal for the cell.

2.1.2. Ionizing radiation damage

Sources of ionizing radiation include X-rays, gamma rays, alpha and beta particles, protons, neutrons, electrons and cosmic rays (13). All of the above have the ability to ionize surrounding matter. As with UV, DNA is considered the

inactivation 'target' molecule of ionizing radiation. In contrast to UV, ionizing radiation directly induces single and double strand breaks which are lethal to a cell (13,33). These DNA strand breaks occur in the sugar-phosphate backbone of the double helix and increase with increasing radiation dose.

Another difference between UV and ionizing radiation damage involves the role of oxygen. No difference in susceptibility is observed between cells that are irradiated with far-UV light in aerobic or anoxic conditions (13). However, bacteria are more sensitive to ionizing radiation when irradiated under aerobic conditions than under an anaerobic environment. The hypothesis is that the O_2 and water molecules are ionized to create hydroxyl radicals which damage the DNA. The result is that a lower radiation dose is needed under aerobic conditions to effect the same amount of cellular killing as that seen in anoxic conditions.

2.1.3. Oxidative damage

In the 1960's there was an increasing interest in the effect of oxygen radical species on organisms. The superoxide anion, hydrogen peroxide and hydroxyl radical are all intermediates of oxidative respiration formed by the sequential reduction of molecular oxygen to water (18). The hydroxyl radical is toxic to many cellular components (18,23,28,31) possessing the ability to oxidize DNA, RNA, lipids, proteins and fatty acids. It is the damage to DNA

however, that is the greatest cause of cell death. Due to the highly reactive nature of the hydroxyl radical, hydrogen peroxide is most commonly used in laboratory experiments examining the effect of oxidative damage on cells (28).

H₂O₂ induces single and double strand breaks in the sugar-phosphate backbone of the DNA molecule (13). The reactive species is most likely the hydroxyl radical, a result of the reduction of H₂O₂.

Imlay and Linn (23) studied the effect of hydrogen peroxide on Escherichia coli and described a bimodal response to oxidative damage. This response demonstrated two modes of susceptibility: mode 1 occurred at low H₂O₂ concentrations (1-5 mM) and mode 2 at high concentrations (greater than 20 mM). They hypothesized that mode 1 killing resulted from DNA damage: E. coli strains deficient in DNA-repair functions were more susceptible to low H₂O₂ concentrations. Also, H₂O₂ partially induced SOS-mediated mutagenesis with a dose-response curve similar to that of the killing curve. These results were interpreted as suggesting an overlap between radiation and oxidative repair mechanisms (29).

2.1.4 Measurement of cellular survival

The inactivation of a population of cells by a particular agent is characterized by a survival curve: a semilog plot representing the surviving fraction of the population as a function of the dose (for example the UV fluence) (33,45,47). There are two important parts of a survival curve. The first

is the area in which any increase in the increment of the applied dose results in a decrease in the population by a constant factor. This relationship will approximate a straight line with a negative slope. The second region of a survival curve occurs at low doses where the slope may approximate zero until some threshold dose level is reached, after which an exponential death rate is observed. This shoulder region of the curve is attributed to the cellular repair of DNA damage.

The equation describing the survival curve (19) is:

$$S/S_0 = e^{-cf}$$

where S_0 is the population count at zero dose, S is the number of survivors at each dose F , and c is the slope of the exponential portion of the curve. A special case involves radiation curves: when the slope and dose are equal, $S/S_0 = e^{-1} = 0.37$. The dose resulting in 37% survival of the population is called the mean lethal dose or fluence if UV is used. This value (D_{37}) expresses the mean number of inactivating hits per biological unit and allows researchers to compare the sensitivities of varied populations of organisms.

2.2. DNA REPAIR

Organisms have developed a number of enzymatic pathways to repair DNA damage caused by radiation and chemical agents. E. coli has been the most extensively studied model for DNA-damage repair and will be the model used here. DNA repair processes can be divided into two major groups: error-free and

error-prone (13,56,58). There are three major types of error-free repair: 1) photoreversal or photoreactivation; 2) excision repair; and 3) recombination or post-replicative repair. Error-prone repair is mediated by recA-lexA interactions which make up the SOS response.

2.2.1. Photoreactivation

The effect of visible light on UV-survival was first observed by Kelner in 1949 (19). Photoreactivation, as the phenomenon is called, involves the cleavage of the pyrimidine dimer to two pyrimidine base monomers (19,45,58). Photolyase, a flavin-containing protein responsible for the reaction, is produced constitutively in E. coli cells. The only known function of this enzyme (19) is to cleave pyrimidine dimers. The molecule first binds to the DNA face containing the cyclobutane ring making contact with phosphates both 5' and 3' to the damage. This initial step is reversible and can take place under dark conditions. Once bound, photolyase utilizes a photon of light to reduce the ring, monomerizing the dimer. Maximal activity requires wavelengths between 300-330 nm.

2.2.2. Excision repair

In contrast to photoreactivation, excision repair can take place in the absence of light. The pathway includes the formation of a protein complex which recognizes the DNA lesion, binds to it and makes two incisions in the damaged strand thus releasing an oligonucleotide containing the dimer. In 1962, Howard-Flanders and Theriot (22) isolated three UV-

sensitive E. coli mutants. These mutations were mapped to three genetically distinct loci labelled uvrA, uvrB and uvrC. The fact that double mutants did not have a greater sensitivity to UV than single mutants suggested that the proteins encoded by the genes formed a reactive complex rather than acting in a sequential fashion.

The preincision complex is formed as follows (54): Two monomers of the UvrA protein form a dimer in an ATP-dependent reaction. The UvrB protein then binds to this complex. The UvrA subunits 'direct' UvrB to the lesion causing localized unwinding and then dissociates from the strand leaving a stable UvrB-DNA complex. This complex is a binding site for two UvrC proteins on either side of UvrB. It is the UvrC protein that effects the dual incision by hydrolyzing the eighth phosphodiester bond 5' and the fourth or fifth bond 3' to the lesion. No incision takes place in the absence of UvrC. Two additional protein factors are required in vitro to excise the lesion and turnover the UvrBC complex; UvrD or helicase II and DNA polymerase I. It is thought that helicase II is involved in the dissociation of UvrC from ssDNA and aids in the binding of DNA polymerase to the 5' incision site to begin resynthesis of the gap using the undamaged strand as a template. The repaired region is then sealed by DNA ligase.

There are many treatments, both pre- and post-irradiation that may affect the efficiency of DNA-repair processes. One such treatment that involves excision repair is called liquid-

holding recovery (LHR). When cells are held in a non-nutrient buffer subsequent to irradiation, their survival rate increases. This observation is generally attributed to the decreased rate of DNA replication which allows the excision repair process a longer time period to eliminate dimers from the DNA strand (19,47).

2.2.3. Post-replication repair

The third mechanism of error-free DNA repair is recombination or post-replication repair. This pathway was discovered through observations that E. coli mutants deficient in homologous recombination also demonstrated an increased sensitivity to UV radiation (18,58). The mutation was mapped to the recA gene, whose product is involved in genetic recombination. Post-replication repair is thought to occur as follows (19,33,47): subsequent to UV irradiation or exposure to other DNA damaging agents, when the replication fork encounters a lesion, the polymerization activity is halted only to reassociate further downstream. This leaves a gap approximately a thousand base pairs long (as opposed to 12-13 bp in excision repair) in the newly synthesized daughter strand across from the dimer. These gaps are filled by homologous sequences from the complementary parental strand in a recombinational process catalyzed by the RecA protein. The resulting parental strand gaps are then filled by DNA polymerase I using the daughter strand as a template.

2.2.4. Relationships between repair mechanisms

To understand the interactions between these error-free repair processes, Moss and Davies (35) in 1974 performed a series of experiments that utilized four strains of E. coli: 1) one that was proficient in both excision and recombination repair (uvr⁺ rec⁺) 2) a mutant deficient in recombination repair (uvr⁺ rec⁻) 3) a mutant that lacked excision repair (uvr⁻ rec⁺) and 4) a double mutant that lacked both types of repair (uvr⁻ rec⁻). All strains were irradiated then subjected to the following conditions: a liquid-holding period (LHR); LHR followed by exposure to photoreactivating light (PR); and PR followed by LHR.

As anticipated, no increase in survival was detected after LHR in the excision repair deficient strains. However if those strains were exposed to PR before or after LHR, the survival levels increased until a plateau was reached. In the (uvr⁺ rec⁻) mutant, an increase in survival was observed after the LHR period, which demonstrated the presence of excision repair. The addition of a PR period either before or after LHR further increased survival.

2.2.5. SOS repair

In addition to error-free or accurate DNA repair; recA⁺ cells are also capable of an error-prone DNA repair mechanism which is believed to be the cause of UV-induced mutagenesis (47,58). Damaged DNA or interruption of DNA synthesis induces a co-protease function of the RecA protein. The inducing

signal is thought to be the binding of RecA to ssDNA. This protease activity cleaves the LexA protein which acts as a repressor for a wide variety of genes. Among the genes regulated by LexA are umuC and umuD. The activity encoded by these genes interferes with the ability of DNA polymerase to distinguish dimers and allows the insertion of bases across from a lesion where normally a gap would be formed (47). Because the lesion is non-instructional, the probability of mispairing is high. Therefore an incorrect base is added across from the lesion which is then propagated through successive DNA replication cycles. This mechanism is referred to as SOS repair since the induction of this function is one of several functions derepressed to aid in cell survival when DNA is damaged (58).

2.2.6. SOS regulatory network

Due to its unique role in DNA metabolism, RecA may play a major role in the development and evolution of natural populations in the environment (32). It has the ability to direct repair and mutagenesis of damage caused by solar radiation, chemical antagonists and possibly oxidative insults; and also may determine and sustain the genetic diversity of natural populations through its recombinase activity (32,47,56,58). In situ studies have detected homologous recombination by conjugation, transduction, and transformation (32). Extrachromosomal genetic material present in plasmids and bacteriophage may serve as reservoirs

of infrequently needed genes. Thus less energy is expended by the cell for genetic maintenance.

Miller and Kokjohn (32) have recently reviewed the evolutionary conservation of recA. RecA analogues with a high degree of homology to the E. coli protein have been found in every prokaryotic species tested. Through complementation studies with E. coli recA mutants, it is known that these analogues have similar biological properties. The universal presence of RecA protein analogues in bacteria that have been studied, and the high degree of conservation with both sequence and function among species and genus suggests that the recA gene developed early in the course of evolution. Miller and Kokjohn (32) speculate that recombination may have arisen as a 'sloppy replication process' in which RecA held the parental strands apart. Also the protein may have had a role in the formation of genomes, assembling transcriptional units for functional proteins.

In E. coli, the RecA protein appears to have a regulatory role in responding to a number of DNA-damaging agents in its environment (56,58). Induction of UV mutagenesis is but one of several physiological responses activated by the co-protease activity of RecA on the LexA protein. Others include: Weigle reactivation and mutagenesis of UV-irradiated bacteriophage, induction of excision repair proteins, and cell filamentation (58). Several other regulons have been identified that are involved in protection of the cell from deleterious agents.

These include the heat response, adaptative response and a genetic network involved with oxidative protection (32,56). There is an apparent overlap between these regulatory systems. For example, the RecA gene product (23,29) and the UvrABC nuclease complex (54,56) which repair radiation-damaged DNA appear to protect E. coli from oxidative damage. Also, H₂O₂ resistant mutants of Salmonella typhimurium constitutively produce several proteins involved in heat-shock resistance (11).

2.3. ECOLOGY OF BACTERIAL RADIORESISTANCE

2.3.1. Natural radiation

The two sources of natural ionizing radiation that reach the surface of the earth come from cosmic radiation and the decay of radioactive metals in the earth's crust (19). Neither amount to levels that have been shown to significantly affect life. Far-UV light (UV-C) and much of the UV-B wavelengths are absorbed by the atmospheric ozone layer. That leaves the near-UV and visible light wavelengths as the major sources of radiation from which organisms must protect themselves (excluding the possibility of low level electromagnetic radiation) (24,37).

2.3.2. Distribution of bacterial radioresistance

The ability to tolerate exposure to radiation has a definite ecological advantage. There is a wide range of radiation susceptibilities found in microorganisms radiation susceptibility has been correlated to the natural habitats

they occupy (24). For example, the *Deinococci* are free-living bacteria commonly found in soils and natural waters where they are constantly exposed to large fluxes of solar radiation (33,34). One can easily argue the advantage these bacteria have by possessing an efficient mechanism to repair radiation damage. *E. coli*, an intestinal bacterium which is far more sensitive to radiation than *D. radiodurans*, may be exposed to intermittent levels of solar radiation as it is transferred between hosts (24). *E. coli* does possess all the major DNA-repair pathways. These mechanisms may be advantageous in the intestinal environment where it must protect itself from DNA-damaging chemical agents.

Stamm and Charon (46) UV irradiated two different *Leptospira* spp.; *L. biflexa* serovar patoc Patoc I, a free-living strain and *L. interrogans* serovar pomona Pomona, a pathogenic strain. They found the free-living strain exhibited a large shoulder at low UV doses suggesting proficiency in DNA repair; while the pathogenic strain exhibited an almost linear survival curve with a slight shoulder. The D_{37} values for *L. biflexa* and *L. interrogans* were 1,450 and 400 $\mu\text{Ws cm}^{-2}$ respectively.

Calkins and Thordardottir (8) exposed a variety of organisms from various habitats to sunlight. These included fresh-water bacteria, yeast, diatoms, algae and arthropods. The conclusion of the study was that the organisms could not tolerate solar radiation levels much greater than those

present in their natural habitats. Commenting on this subject, Jagger (24) postulated that "...microorganisms expend no more energy than is absolutely necessary to resist the inactivating effects of solar radiation."

2.3.3. Deinococcus radiodurans

A wide range of bacteria, viruses, fungi and other eukaryotic cells have been tested for their responses to radiation. The most radiation resistant group of bacteria are the Deinococci. There are four species in the genus of which D. radiodurans is the type species (36). Originally recovered from gamma irradiated cans of meat; D. radiodurans is a gram positive, non-sporeforming, red-pigmented bacterium (33).

In addition to their high degree of radiation resistance, the Deinococci are related on the basis of their unusual cell wall characteristics and their mode of cell division (33,36).

Cells may be present in groups of 2/4 to 16/32. Cell division occurs on two planes resulting in a sheet of cells which may break off due to shearing forces. Chou and Tan (10) have recently shown a correlation between salt concentration and persistence of D. radiodurans in the larger groups. No correlation has been demonstrated between radioresistance and the multi-cellular forms however (33).

The resistance of D. radiodurans to radiation is apparently due to a high efficiency of excision repair (33). Data point to the presence of two endonucleases that can incise the DNA lesions prior to excision. An interesting

finding is that photoreactivation is absent in the wildtype strain (19). Similar results have been found in mutants lacking excision repair which eliminates the possibility of the excision repair mechanism masking weak photoreactivating activity (33).

2.3.4. Morphology and radiation protection

In addition to DNA-repair capacity, physical screening and interference with energy particles play a role in radiation protection. Gram positive bacteria which possess thicker cell walls are generally more UV resistant than gram negative cells; and bacterial spores are more resistant than vegetative cells due to their thick spore coat (24).

Pigments, especially carotenoids, have been hypothesized to serve as free radical quenchers (57) and therefore may provide protection from agents such as near UV/solar radiation, and the effects of photosensitizing molecules such as porphyrins (51). However, the ability of pigments to protect against ionizing radiation and far UV light have not been firmly established (20,33,51). For example, all four radioresistant Deinococcus strains exhibit red pigmentation (36). However, colorless mutants of D. radiodurans do not demonstrate a loss of radiation resistance (33). Tuveson et al (51) cloned an E. coli strain that expressed carotenoid pigments derived from Erwinia hervicola and exposed it to near UV light and a number of photosensitizers. The survival patterns suggested that pigmentation protected against

radical-induced damage to the cell membrane but did not influence DNA damage.

There is a correlation between organisms living in habitats exposed to intense sunlight and pigmentation. Hermansson et al (20) found a significant increase in the number of pigmented bacteria living at the air-water interface off the coast of Sweden compared to bacteria in the bulk water column. At the air-water interface, 8.7% of all isolates were yellow and 47.4% were red, pink or brown. Many red and pink pigmented organisms are associated with salt lakes. The red pigment of the alga Dunaliella salina increases with increasing salinity and UV flux as seasonal evaporation occurs (49). The halophilic bacterium Halobacterium cutirubrum, a red pigmented gram negative rod, demonstrated UV resistance 800x that of E. coli B (60). In that study the authors also hypothesize that carotenoid pigments may aid in photoreactivating efficiency by transferring light energy to the photolyase enzyme.

2.4. THE SUBSURFACE ENVIRONMENT

Concern over the contamination of aquifers from the deep injection of organic and mixed wastes (38,39,42,59) has spurred interest into the nature of the deep subsurface environment. Since the mid 1980's the Department of Energy (51,52,59) has funded research to study subsurface microbial ecology and investigate the potential of subsurface microorganisms in the bioremediation of toxic organic

compounds and radionuclides (16).

The drilling technologies necessary for recovery of microbiologically suitable subsurface sediments has been available the last 5 years (39,40). From 1986-1988 subsurface sediments were obtained from boreholes located at the Savannah River Plant (SRP) and from a site at nearby Allendale, SC (42). The sites were located in the Upper Atlantic Coastal Plain and included interspersed layers of unconsolidated sands, clayey sands and sandy clays. These sediments were deposited during the Cretaceous period approximately 66-100 million years ago. Groundwater moving through these sediments originate from recharge areas 60 to 80 km upland and have been dated at least 3000 to 4000 years old (42).

2.4.1. Subsurface microbiology

The drilling techniques utilized to recover subsurface soil samples have been detailed by Phelps et al (39) and Phelps and Russell (40). A number of quality assurance analyses were employed to monitor the contamination of samples with drilling fluid. These included rhodamine dye, potassium bromide and perfluorocarbons.

Examinations of the SRP sediments have revealed surprisingly diverse microbial populations (3,4,16,39,44). Balkwill et al (4) reported the presence of 10^5 - 10^8 viable cells/g soil. Comparison of colony forming units with direct microscopic counts showed that up to 100% of cells were viable in some samples (44). This observation is in contrast to

results normally observed from surface soils in which approximately 10% of the cells present are viable. The sediments were dominated by aerobic heterotrophic bacteria. While 82% of the bacterial isolates could use glucose aerobically, only 4% could ferment it (4). Benoit and Phelps (5) reported that at a depth of 463 m, up to 10% of the total population of bacteria of the Allendale site was microaerophilic. They hypothesized that microaerophily was an evolutionary advantage in the low-oxygen oligotrophic environment. Chapelle and Lovely (9) showed that the rate of [2-¹⁴C]acetate and [U-¹⁴C]glucose metabolism by subsurface bacteria was among the slowest ever measured. In general, several studies (3,9,30) found both bacterial numbers and metabolic rates were lower in clay sediments than in the unconsolidated sands.

Subsurface bacteria utilize a wide range of substrates. In addition to the aerobic (3,21), microaerophilic (5), and anaerobic (26,30) utilization of simple carbon compounds, subsurface bacteria have been shown to degrade a variety of organic wastes. Fliermans et al (17) demonstrated the degradation of TCE using a bacterial consortium enriched from subsurface sediments. Brockman et al (6) isolated two subsurface gram negative bacteria capable of metabolizing quinoline both aerobically and anaerobically.

2.5. OBJECTIVE

Initial studies of the subsurface environment have

revealed a diverse and metabolically active microbial community. However, much of the ecology and physiology of these microorganisms is not understood. Subsurface bacteria represent a potential reservoir of organisms capable of performing the bioremediation of organic and mixed wastes in oligotrophic environments. The investigation of DNA-repair potential in deep subsurface bacteria may aid in identifying microorganisms for further use.

The objective of this work was to examine the level of DNA-damage tolerance in deep subsurface bacteria as compared to surface bacteria. To accomplish this task, two groups of subsurface bacteria, aerobic and microaerophilic isolates, and reference bacterial cultures were exposed to UV light, gamma radiation and H_2O_2 . Resistance data in the form of survival curves, regression data and D-values were used to evaluate bacterial susceptibilities to DNA-damage. In addition to cell survival, some subsurface isolates were assayed for photoreactivating activity and for the presence of an SOS-like response.

Bacterial survival curves from each of the three treatments were compared to determine the feasibility of substituting practical DNA-damage assays such as UV and H_2O_2 for ionizing radiation studies.

3.0. MATERIALS AND METHODS

3.1. BACTERIAL ISOLATION PROCEDURES

3.1.1. Subsurface bacteria

Subsurface soil samples were obtained through the deep subsurface science program of the Department of Energy and Savannah River Laboratories. A thorough description of the drilling sites and protocols have been detailed elsewhere (39,40). Soil samples from depths of 150-500 m served as the source for primary isolation of bacterial colonies. The isolation medium (5) was Dilute-Substrate Mineral Salts (DSMS). Pure colonies were isolated from soil-inoculated MPN tubes which contained either DSMS broth, DSMS semi-solid media (DSMS + 1.5 g L⁻¹ Agar Noble [Difco]) or from agar plates (DSMS broth + 15 g L⁻¹ Agar Noble).

Initial incubations at 25°C required over 1 week before macroscopic growth was observed. Subsequent transfers of pure colonies resulted in consistent colony formation ranging from fast-growing strains in 48 h to slower growing strains in 1 week. Bacterial colonies were randomly selected and included gram positive and gram negative, pigmented and non-pigmented, aerobic and microaerophilic isolates. Microaerophilic strains were identified based on characteristic banding properties when inoculated into DSMS semi-solid agar tubes and the demonstration of spreading motility on aerobically incubated DSMS agar plates (5).

A total of 39 aerobic and 24 microaerophilic subsurface bacterial isolates were screened for UV resistance in this study. All subsurface isolates were maintained aerobically on DSMS plates at 25°C.

3.1.2. Surface soil bacteria

Surface soil bacterial colonies were isolated from a Knoxville, TN site. The soil samples were aseptically collected from a depth of 3 cm. The isolation and maintenance medium for the surface soil isolates was DSMS agar plates as described above. A total of 31 surface cultures were tested for UV resistance including aerobic gram positive, gram negative, pigmented and non-pigmented colonies.

3.1.3. Reference bacterial strains

Reference bacterial strains used as controls in the study are listed in Table 2. All reference bacteria were maintained at 30°C on T-soy agar (Difco) plates.

3.2. UV IRRADIATION PROCEDURES

3.2.1. UV apparatus

Samples were UV irradiated in a 46 x 15 x 30 cm foil-lined plexiglass box which contained a 15W, 254nm UV light source (NIS germicidal lamp) affixed at the top. The light source was positioned 34 cm from the samples. The UV fluence rate was measured with a UVX Radiometer (UVP Inc., San Gabriel, CA) in units of $\mu\text{W cm}^{-2}$ per s. The fluence rate was modified by positioning the

Table 1. List of reference bacterial strains.

<u>Name</u>	<u>ATCC #</u>
<u>Bacillus cereus</u>	14579
<u>Bacillus subtilis</u>	6051
<u>Deinococcus radiodurans</u>	13939
<u>Escherichia coli</u>	9637
<u>Escherichia coli</u>	25922
<u>Escherichia coli</u> B	---- ^a
<u>Escherichia coli</u> B/r	---- ^a
<u>Micrococcus luteus</u>	10240
<u>Pseudomonas aeruginosa</u>	15692
<u>Pseudomonas fluorescens</u>	13525
<u>Staphylococcus aureus</u>	12600

^aStrain obtained from the VPI & SU collection.

samples along the length of the box. There was no detectable deviation in the fluence rate over time. The highest fluence was measured in the center of the box with decreasing fluence rates towards the ends. Total UV dose was determined by time of exposure to the UV fluence rate in units of $\mu\text{Ws cm}^{-2}$.

All UV irradiation procedures were performed under red light to prevent photoreactivation from occurring.

3.2.2. UV irradiation of cultures in phosphate buffer

Subsurface and surface soil stationary-phase bacterial isolates grown in modified DSMS broth, and stationary-phase reference bacteria grown in T-soy broth were centrifuged for 15 min at $5000 \times g$, washed twice with 0.2 M phosphate buffer (pH 7.0) (14) and resuspended in the buffer to a final concentration of $10^6 - 10^8$ cells ml^{-1} . To avoid spore formation in the Bacillus strains, log-phase cultures were irradiated. A 2 ml aliquot of each culture suspension was transferred to a 9 mm petri plate resulting in a depth of less than 3 mm of liquid. The open petri plates were exposed to a UV fluence rate of $700 \mu\text{W cm}^{-2}$ per s resulting in total doses of $0 - 28,000 \mu\text{Ws cm}^{-2}$ using the apparatus described above. Each bacterial suspension was irradiated individually, and was manually agitated to prevent settling of the cells.

Following irradiation, a 1.0 ml aliquot of each suspension was serially diluted in buffer and plated using the spread-plate technique. The medium for subsurface and surface soil isolates was modified DSMS agar and the reference

bacteria were plated onto T-soy agar (Difco). The plates were wrapped in foil and incubated for up to ten days. Incubation temperature was 25°C for soil isolates and 30°C for reference bacteria. Percent survival at each dose was determined by comparing colony counts of irradiated cells to a non-irradiated control.

UV resistance by this method was defined as the demonstration of a $\geq 1.0\%$ survival level at a UV dose of $1.40 \times 10^4 \mu\text{Ws cm}^{-2}$. At this dose, stationary-phase E. coli B cells exhibited a survival level of approximately 0.0001% (data not shown).

3.3. ASSAY FOR PHOTOREACTIVATION

Log-phase bacterial cultures were washed, suspended in phosphate buffer and UV irradiated as described previously with the following modifications: aerobic subsurface isolates and D. radiodurans were exposed to a UV fluence of $500 \mu\text{W cm}^{-2}$ per s; while microaerophilic subsurface isolates and E. coli strains were exposed to a fluence of $300 \mu\text{W cm}^{-2}$ per s.

Immediately following irradiation, the bacterial suspensions were diluted and replicate sets of each bacterial culture were plated as described above. One set of plates was immediately wrapped in foil and incubated for up to 10 days. The second set of covered plates was placed in a 46 x 24 x 24 cm foil-lined box and exposed to a 360 nm photoreactivating light source (Multi-ray lamp) positioned

approximately 20.5 cm above the samples. The fluence was measured at $200 \mu\text{W cm}^{-2}$ per s by a UVP radiometer. Exposure time to PRL was 30 min for D. radiodurans and the aerobic subsurface isolates; 20 min for E. coli and the microaerophilic subsurface isolates. The plates were incubated as described above and the percent survival was calculated for each isolate with and without PRL.

3.4. ASSAY FOR SOS RESPONSE

Four log-phase subsurface bacterial isolates (two aerobic and two microaerophilic) were suspended in buffer as previously described. Prior to UV irradiation the suspensions were exposed to a sublethal dose of approximately $60 \mu\text{M H}_2\text{O}_2$ in 15 ml screw-capped tubes. Time of exposure was 15 min. Following H_2O_2 exposure, the cell suspensions were UV irradiated as described in the photoreactivation experiment; the aerobic isolates irradiated with a fluence of $500 \mu\text{W cm}^{-2}$ per s and the microaerophiles with $300 \mu\text{W cm}^{-2}$ per s. The cell suspensions were then diluted and plated in the dark as described previously.

3.5. GAMMA IRRADIATION

A ^{60}Co ionizing radiation source located at Oak Ridge National Laboratory (Oak Ridge, TN) was utilized to test the survival of subsurface bacteria to ionizing radiation. The activity of the source was 4.1 kCi with a dose rate of 350 krad h^{-1} .

Log-phase bacterial cultures to be irradiated were grown

as previously described. The cultures were then centrifuged for 30 min at 3000 x g, washed twice and resuspended in phosphate buffer. Two ml aliquots of the suspensions were transferred to 15 ml screw-capped tubes and placed on ice in the dark for transportation to the radiation source.

The irradiation procedures were carried out by ORNL personnel. The cultures were irradiated in groups of 7 or 8 closed screw-capped tubes. The time of exposure ranged from 0 - 120 min. Immediately following irradiation, the tubes were placed on ice and in the dark until the diluting and plating procedures could be accomplished. The average time between irradiation and plating was 2 h. The incubation times and temperatures were identical to those described previously. Percent survival was determined at each exposure time.

3.6. H₂O₂ CHALLENGE

Log phase bacterial isolates to be challenged with H₂O₂ (30% Mallinkrodt) were centrifuged, washed and resuspended in buffer as described previously. A 1.27 M H₂O₂ working solution was made by diluting H₂O₂ with distilled deionized water and was stored at 4°C. A fresh working solution was made for each experiment. Appropriate μ l volumes of H₂O₂ for concentrations of 0 - 640 mM H₂O₂ were added to 15 ml screw-capped tubes containing media and a 0.5 ml aliquot of the cell suspension for a total volume of 5 ml. Subsurface isolates were tested in modified DSMS broth; E. coli and D. radiodurans were tested in T-soy broth. Cells were challenged with each H₂O₂ dose for

15 min. Following exposure, 1.0 ml aliquots were diluted in phosphate buffer, plated and incubated as described above.

3.7. CALCULATIONS

3.7.1. D-value calculations

To compare the sensitivities of unrelated bacterial strains to DNA-damaging treatments, D_{37} , D_{10} and D_1 values were calculated. D-values are a measure of the quantity (or dose) of a toxin needed to attain a certain percent of cell survival. For example, sensitivity to radiation is often described by the D_{37} value (19,33). This parameter is defined as the radiation dose required to inactivate 63% of a bacterial population, or that required to kill one viable unit.

D-values were calculated as described by Harm (19). The equation utilized for a non-shouldered survival curve was:

$$S/S_0 = e^{-cF} \quad (3.1)$$

where S/S_0 was the percent survival at dose F ; and $-c$ was the slope of the exponential portion of the curve. Therefore, if the slope is known, the D-value for any percent survival S/S_0 is F .

The equation for the exponential part of a shouldered survival curve was:

$$n = e^{F_T/F} \quad (3.2)$$

where n was the ordinate axis intercept of the straight line of the exponential portion of the curve; F_T was the threshold dose (a measure of the length of the shoulder); and F was the

dose calculated in equation 3.1 for percent survival S/S_0 . The D-value, then, is the sum of F and F_T (33).

3.7.2. Fluence reduction factor

Certain treatments such as exposure to photoreactivating light modify the efficiency of the inactivating effect of UV light on bacteria. The influence of these treatments on cell survival can be measured by the fluence reduction factor (FRF) (19). The FRF is defined as the ratio of a UV-induced effect (i.e. D_{37} value) when the treatment is applied over the effect without the treatment. In this study, FRF values were utilized to quantify the influence of photoreactivating light and a sublethal dose of H_2O_2 on bacterial survival to UV light.

4.0. RESULTS

4.1. UV IRRADIATION OF REFERENCE BACTERIA

UV resistance data for reference bacterial strains exposed to 254 nm UV radiation are shown in Fig. 1 and Tables 2 & 3. The most resistant bacterium tested was D. radiodurans. The survival curve for this bacterium exhibited a large shoulder (Fig. 1) with a slope of -0.05 (Table 2). The D_{37} and D_{10} values (Table 3) were $5.7 \times 10^4 \mu\text{Ws cm}^{-2}$ and $1.3 \times 10^5 \mu\text{Ws cm}^{-2}$ respectively. The D_{37} value obtained in this study was in agreement with previously reported D_{37} values for D. radiodurans (Table 4).

Comparisons of D-values between gram positive and gram negative bacteria show that the gram positive isolates were more resistant to UV light. Three of five gram positive bacteria exhibited a shoulder region at the low UV dose range indicated by a positive intercept number. Only one gram positive isolate, S. aureus had D-values lower than a gram negative bacterium. The survival curves for three E. coli strains and other gram negative bacteria were steeper than most of the gram positive strains and all lacked a shoulder. For example, E. coli B (slope, -0.96) and E. coli B/r (slope, -0.70) demonstrated higher sensitivities to UV than M. luteus (slope, -0.50) and B. subtilis (slope -0.44). However, the slopes of the B. cereus and S. aureus inactivation curves were identical at -0.66; similar to that of E. coli B/r and P.

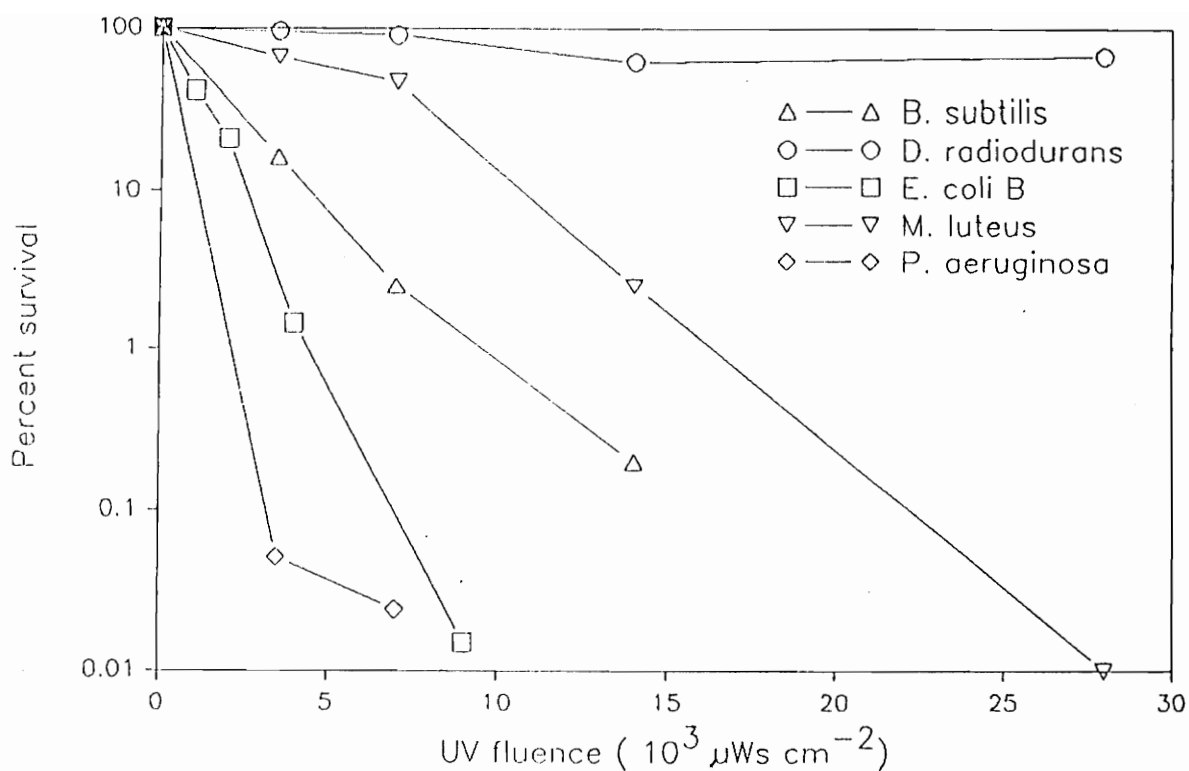


FIG. 1. Survival curves of some reference bacteria irradiated by 254 nm UV light. Phosphate buffered suspensions of stationary-phase cells were irradiated with the exception of *B. subtilis* which was irradiated in log-phase to avoid spore formation. The percent survival was plotted as a function of UV fluence.

TABLE 2. The intercept and slope values of the UV survival curves of reference bacteria. The intercept and slope were calculated from the plot of the natural logarithm of the percent survival as a function of UV fluence.

Organism	ATCC #	Intercept	Slope
<u>D. radiodurans</u>	13939	1.9	-0.05
<u>M. luteus</u>	10240	2.5	-0.50
<u>B. subtilis</u>	6051	-0.22	-0.44
<u>B. cereus</u>	14579	0.37	-0.66
<u>P. fluorescens</u>	13525	-2.0	-0.66
<u>S. aureus</u>	12600	-1.5	-0.66
<u>E. coli</u> B/r	----- ^a	-2.3	-0.70
<u>P. aeruginosa</u>	15692	-0.14	-0.73
<u>E. coli</u> B	----- ^a	-1.7	-0.96
<u>E. coli</u>	9637	-0.36	-1.5

^aObtained from the VPI & SU microbiology collection.

TABLE 3. D-values ($10^3 \mu\text{Ws cm}^{-2}$) of reference bacterial isolates exposed to UV radiation.

Organism	ATCC #	D_{37}^a	D_{10}	D_1
<u>D. radiodurans</u>	13939	57	130	260
<u>M. luteus</u>	10240	7.0	16	32
<u>B. subtilus</u>	6051	2.3	5.2	10
<u>B. cereus</u>	14579	2.1	4.8	9.6
<u>P. fluorescens</u>	13525	1.5	3.5	7.0
<u>S. aureus</u>	12600	1.5	3.5	7.0
<u>E. coli</u> B/r	----- ^b	1.4	3.3	6.6
<u>P. aeruginosa</u>	15692	1.4	3.1	6.3
<u>E. coli</u> B	----- ^b	1.1	2.4	4.8
<u>E. coli</u>	9637	0.7	1.6	3.1

D-value was defined as the UV fluence which reduced a cell population to a specified percentage of the original number of cells. The D-values were calculated from the regression line of the exponential slope of the survival curve as described in Materials and Methods.

^bObtained from the VPI & SU microbiology collection.

TABLE 4. Literature D-values ($10^3 \mu\text{Ws cm}^{-2}$) for microorganisms exposed to UV light.

Organism	D ₃₇	D ₁₀	D ₁	Reference
<u>Bacteria</u>				
<u>Bacillus subtilis</u>	----	7.00	----	31
<u>Deinococcus radiodurans</u>	----	198.60	----	31
	57.00	----	----	34
	50.40	----	----	50
	60.00	----	----	55
<u>D. proteolyticus</u>	89.60	----	----	50
<u>D. radiophilus</u>	67.00	----	----	50
<u>D. roseus</u>	51.30	----	----	50
<u>E. coli</u>	----	2.11	----	2
	----	3.00	----	31
<u>E. coli AB1157</u>	----	----	15.20	43
<u>Legionella pneumophila</u>	----	0.92	1.84	2
<u>Leptospira biflexa</u>	1.45	2.30	3.75	46
<u>L. interrogans</u>	0.40	0.75	1.25	46
<u>Micrococcus luteus</u>	----	19.70	----	31
	10.90	----	----	33
<u>Mycobacterium</u>				
<u>tuberculosis</u>	----	6.20	----	31
<u>Pseudomonas aeruginosa</u>	----	3.60	----	31
	----	5.50	----	31
<u>P. fluorescens</u>	----	3.50	----	31
<u>Salmonella typhi</u>	----	2.14	----	2
<u>Serratia marcescens</u>	----	2.40	----	31
<u>S.marcescens CV</u>	----	10.00	----	27
<u>Staphylococcus aureus</u>	----	2.60	----	31
	----	5.58	----	31
<u>Protozoa</u>				
<u>Giardia lamblia</u>	----	----	>63.00	41
<u>Yeast</u>				
<u>Candida albicans</u>	----	44.74	----	31

aeruginosa (slope, -0.73). The survival curves for gram negative bacteria were linear exponential slopes with an absence of a shoulder region. The majority of the gram positive bacteria demonstrated a shoulder region at lower UV fluences generally indicated by a positive intercept value. The presence of a shoulder increased the calculated UV resistance value for a bacterium above that calculated solely from the slope. For example, the identical slopes of B. cereus and S. aureus each yielded an F value at 37% survival of $1.5 \times 10^3 \mu\text{Ws cm}^{-2}$. However, the survival curve of B. cereus possessed a small shoulder with an F_1 of $0.56 \times 10^3 \mu\text{Ws cm}^{-2}$. Therefore, the D_{37} of B. cereus was $2.1 \times 10^3 \mu\text{Ws cm}^{-2}$ compared to a D_{37} of $1.5 \times 10^3 \mu\text{Ws cm}^{-2}$ for S. aureus.

4.2. UV IRRADIATION OF SUBSURFACE AND SURFACE BACTERIA

Data describing the distribution of UV resistance in soil bacteria are shown in Table 5. The numbers of UV resistant subsurface and surface soil bacterial isolates were similar: 30.8% and 25.8% respectively. In contrast to aerobic subsurface bacteria, all of the microaerophilic subsurface bacteria tested were UV sensitive; that is, they demonstrated a survival rate of <1.0% at $14,000 \mu\text{Ws cm}^{-2}$.

A relationship between the resistance of bacteria to UV and gram reaction was observed which was also reflected in the reference strains. Among the resistant subsurface aerobic bacteria 83.3% were gram positive; and 62.5% of the surface

TABLE 5. Distribution of the UV resistance trait in stationary-phase soil bacteria. Soil isolates were grouped according to oxygen response, soil horizon location, and presence or absence of pigmentation. Also shown is the percent of UV resistant isolates that demonstrated a gram positive stain reaction.

	Surface aerobic isolates	Subsurface aerobic isolates	Subsurface microaerophilic isolates
	UV resistant ^a /total		
Pigmented:	4/5	10/16	---- ^b
Non-pigmented:	4/26	2/23	0/24
Total screened:	8/31	12/39	0/24
% of UV resistant isolates that demonstrated a positive gram reaction:	62.5	83.3	---- ^c

^aUV resistance defined as a $\geq 1.0\%$ survival rate at a dose of $1.4 \times 10^4 \mu\text{Ws}/\text{cm}^2$ 254 nm light.

^bAll microaerophilic subsurface bacteria were non-pigmented.

^cAll microaerophilic subsurface bacteria were gram negative.

soil isolates resistant to UV were gram positive. A second observation was the predominance of UV resistance among pigmented bacteria. Among the aerobic subsurface bacteria exhibiting pigmentation, 62.5% were UV resistant; and 80% of the pigmented surface soil bacteria were UV resistant. All of the UV sensitive microaerophilic subsurface bacteria were gram negative and non-pigmented.

The survival curves of the three most UV resistant aerobic subsurface bacteria (strains UV1 - UV3) and three representative microaerophilic subsurface bacteria (M1 - M3) are shown in Fig. 2. The UV regression data and D-values for these subsurface bacteria are shown in Tables 6 & 7. The most UV resistant subsurface isolate, strain UV1, exhibited an inactivation slope of -0.42 with a shoulder region (F_1 , $1.2 \times 10^4 \mu\text{Ws cm}^{-2}$). This bacterium was an aerobic, gram positive, orange-pigmented coccus. The D_{37} for this isolate was $1.5 \times 10^4 \mu\text{Ws cm}^{-2}$, lower than D. radiodurans, but twice that of M. luteus ($7.0 \times 10^3 \mu\text{Ws cm}^{-2}$). Strains UV2 and UV3 were also gram positive, pigmented bacteria. The D_{37} of UV2, a yellow-pigmented rod, was $9.6 \times 10^3 \mu\text{Ws cm}^{-2}$ and UV3, a red pigmented coccus had a D_{37} of $8.3 \times 10^3 \mu\text{Ws cm}^{-2}$. These isolates also possessed shoulders at the lower range of UV dose.

All three microaerophilic subsurface bacteria exhibited non-shouldered, linear survival curves. The UV sensitivity of these isolates as shown by their D_{37} values were lower than the

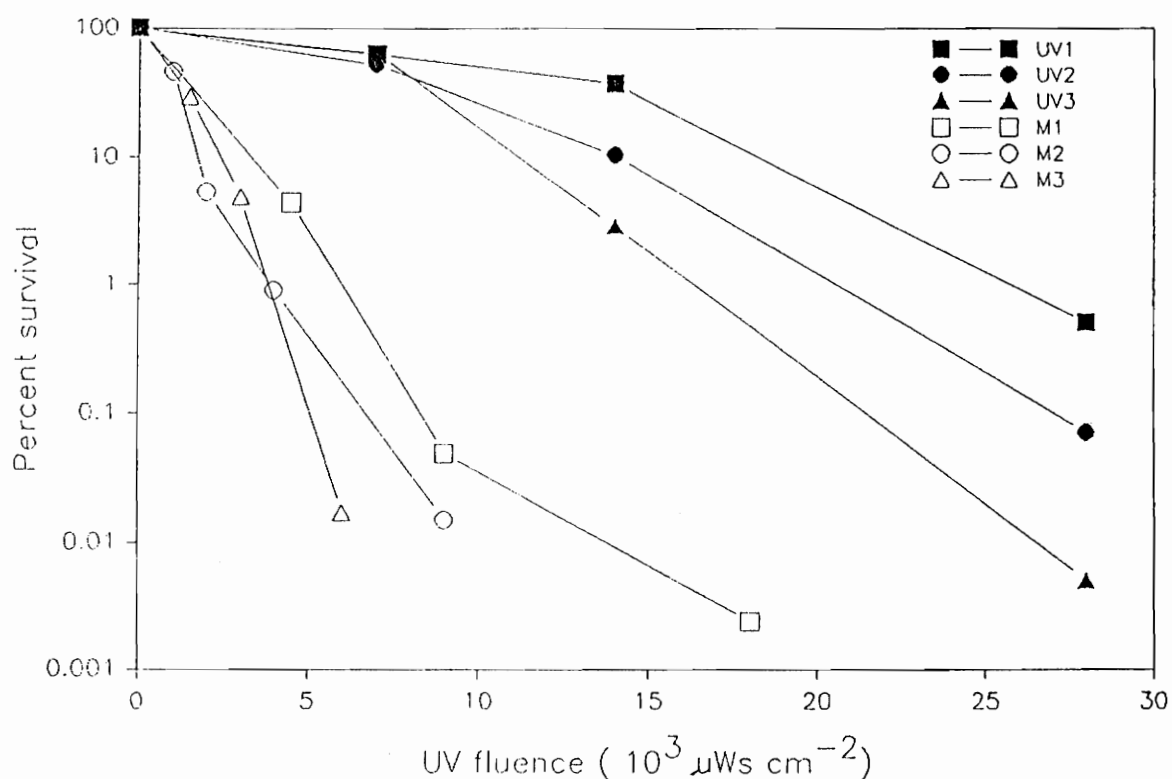


FIG. 2. Survival curves of the 3 most resistant aerobic subsurface isolates (UV1, UV2, and UV3) and 3 representative microaerophilic subsurface isolates (M1, M2, and M3) irradiated by 254 nm UV light. Phosphate buffered suspensions of stationary-phase cells were irradiated. The percent survival was plotted as a function of UV fluence.

TABLE 6. Intercept and slope values of UV survival curves of six subsurface bacteria. The intercept and slope were calculated from the plot of the natural logarithm of the percent survival as a function of UV fluence.

Organism	Intercept	Slope
Aerobic isolates:		
UV1	5.2	-0.42
UV2	2.7	-0.38
UV3	2.4	-0.41
Microaerophilic isolates:		
M1	-0.01	-0.54
M2	-0.26	-1.4
M3	-0.94	-0.68

TABLE 7. D-values ($10^3 \mu\text{Ws cm}^{-2}$) of subsurface bacteria exposed to UV light.

Organism	D_{37a}	D_{10}	D_1
Aerobic isolates:			
UV1	15	34	57
UV2	9.6	22	44
UV3	8.3	19	38
Microaerophilic isolates:			
M1	1.9	4.3	8.6
M2	0.73	1.7	3.3
M3	1.5	3.4	6.8

^aD-values were defined as the UV fluence needed to reduce a cell population to a specified percentage of the original number of cells. The D-values were calculated from the regression line of the exponential slope of the survival curve as described in Materials and Methods.

aerobic subsurface isolates but were similar to the gram negative reference bacteria. One isolate, strain M1 possessed D-values higher than all the gram negative cultures and S. aureus.

4.3. PHOTOREACTIVATION MEASUREMENTS

When E. coli B was exposed to photoreactivating light (PRL) following UV irradiation, the D_{37} was $2.4 \times 10^3 \mu\text{Ws cm}^{-2}$ compared to a D_{37} of $1.8 \times 10^3 \mu\text{Ws cm}^{-2}$ in the absence of PRL (Table 8). This resulted in a fluence reduction factor (FRF) of 0.75. Therefore, UV light was only 75% as effective in inactivating the cell population. D. radiodurans did not possess a photoreactivating mechanism. The D_{37} values for this organism when exposed and not exposed to PRL were identical at $57 \times 10^3 \mu\text{Ws cm}^{-2}$. Therefore FRF of this organism was 1.0.

Survival curves of aerobic and microaerophilic subsurface bacteria exposed to 365 nm PRL following UV irradiation are shown in Figs. 3 & 4. High levels of DNA damage could mask the recovery effect of PRL; therefore, the UV fluence ranges were selected to achieve a 3 - 4 log decrease in cell survival.

A recovery effect was observed when UV3 was exposed to PRL (FRF, 0.12). However, UV1 did not show any evidence of photoreactivation with an FRF of >1.0 which indicated a decrease in cell survival when exposed to PRL. Both microaerophilic isolates demonstrated photoreactivation with FRF values of 0.73 and 0.58 for M1 and M3 respectively.

TABLE 8. D_{37} ($10^3 \mu\text{Ws cm}^{-2}$) and FRF values of UV irradiated bacteria with and without subsequent exposure to 365 nm photoreactivating light (PRL).

Organism	D_{37}^a without PRL	D_{37} with PRL	FRF ^b
<u>D. radiodurans</u>	57	57	1.0
Aerobic subsurface isolates:			
UV1	8.0	4.9	1.6
UV3	10	82	0.12
<u>E. coli</u> B	1.8	2.4	0.75
Microaerophilic subsurface isolates:			
M1	1.6	2.2	0.73
M3	1.1	1.9	0.58

^a D_{37} value defined as the UV fluence which reduced a cell population to 37% of the original number of cells. The D_{37} was calculated from the regression line of the exponential slope of the survival curve as described in Materials and Methods.

^bThe fluence reduction factor (FRF) was defined as the ratio of the D_{37} value when PRL was applied to the D_{37} value without PRL.

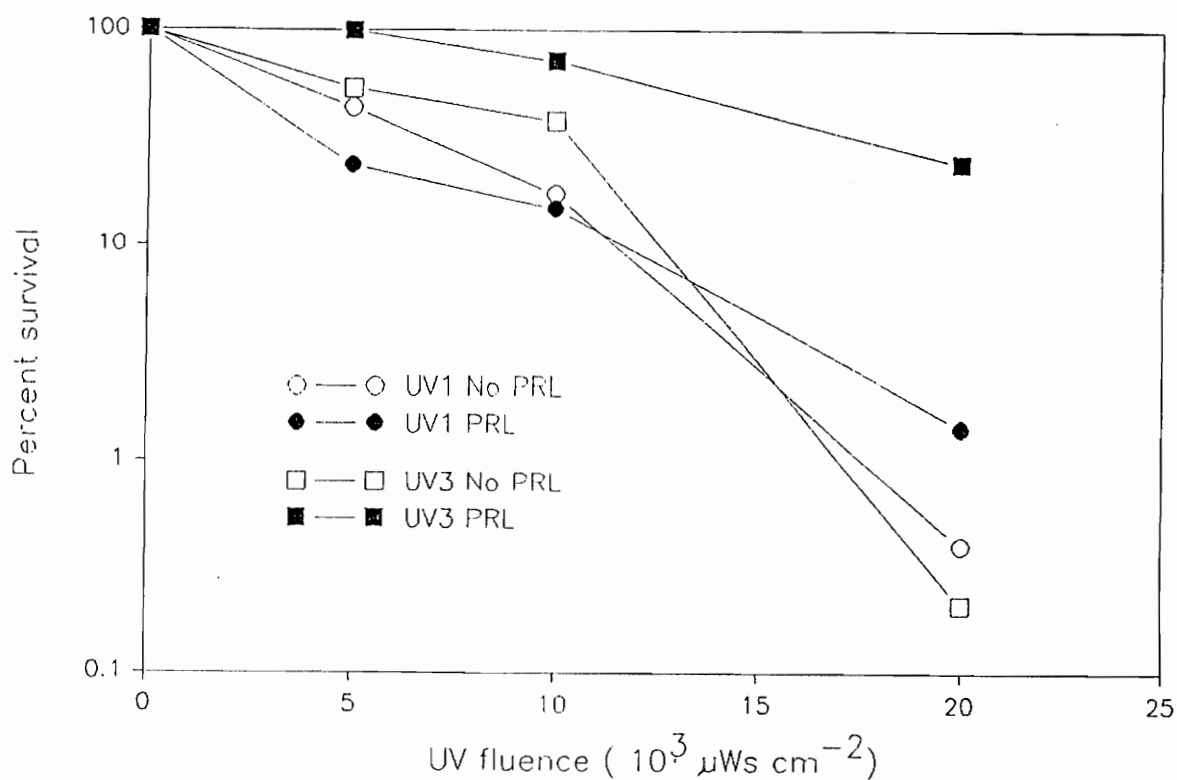


FIG. 3. UV survival curves of aerobic subsurface isolates UV1 and UV3 with and without a 30 min exposure to 365 nm PRL following irradiation. The percent survival was plotted as a function of the UV fluence.

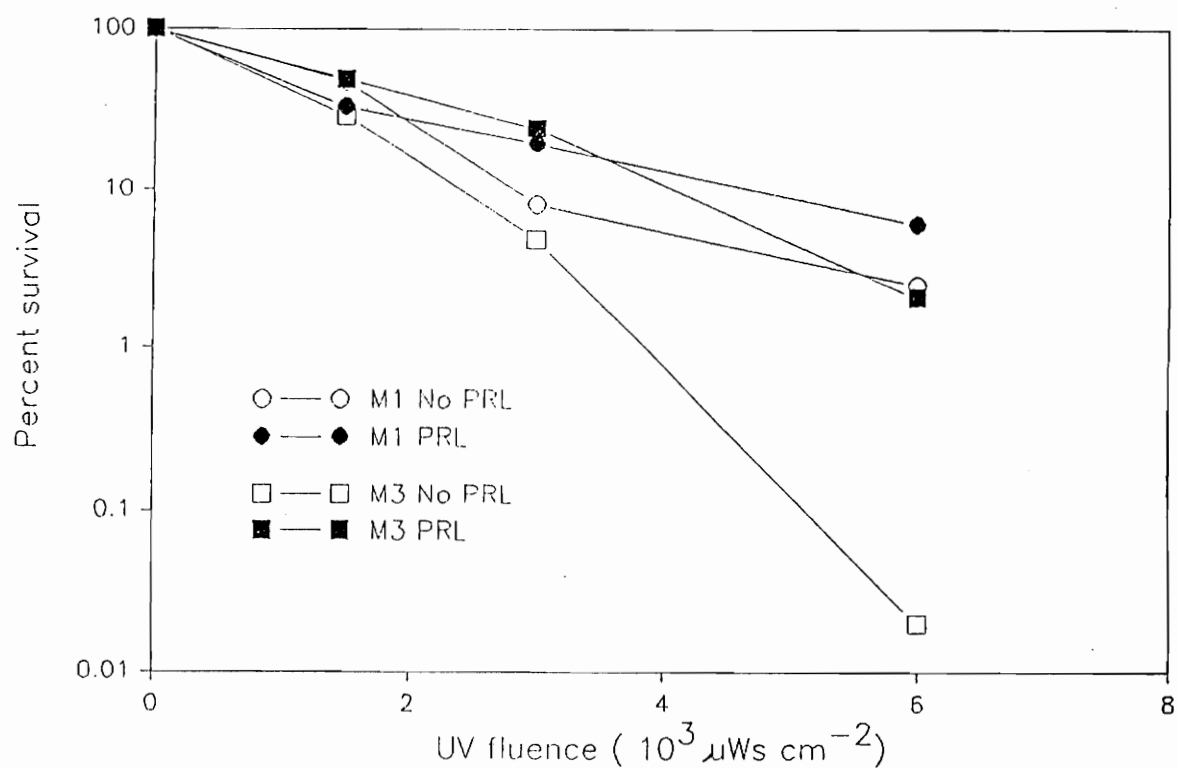


FIG. 4. UV survival curves of microaerophilic subsurface isolates M1 and M3 with and without a 30 min exposure to 365 nm PRL following irradiation. The percent survival was plotted as a function of the UV fluence.

4.4. RESULTS OF SOS RESPONSE INDUCTION

The aerobic and microaerophilic subsurface isolates responded differently to the treatment of sublethal of 60 μM H_2O_2 prior to UV irradiation (Table 9). The microaerophilic isolates M1 and M2 exhibited increased survival after exposure to H_2O_2 with FRF values of 0.70 and 0.79 respectively. The aerobic isolates however, did not demonstrate increased resistance. UV3 exhibited no effect from H_2O_2 (FRF, 1.0) and UV1 displayed a sensitization to UV light subsequent to H_2O_2 exposure (FRF, 1.1).

4.5. GAMMA IRRADIATION OF BACTERIA

The ionizing resistance data of 6 subsurface isolates, D. radiodurans and E. coli ATCC 25922 is shown in Tables 10 & 11. The order of resistance to gamma radiation was similar to that found with UV radiation. D. radiodurans was the most resistant bacterium with a D_{37} of 490 krad, 40x more resistant than strain UV1 (D_{37} , 12 krad). The aerobic subsurface isolates were more resistant to gamma radiation than were the microaerophiles. However, within the aerobic group, the UV3 strain was the most resistant to gamma followed by UV2 and UV1; this is the reverse of the order observed with UV irradiation. A plot of the survival curves (Fig. 5) shows UV1 has a small shoulder at the initial doses which evolves into a steep slope (-0.33) as the dose increases. In contrast to UV1, UV2 and UV3 do not exhibit shoulders but have more gradual slopes (-0.07 and -0.08 respectively) resulting in

TABLE 9. D-values $10^3 \mu\text{Ws cm}^{-2}$) and FRF of irradiated subsurface bacteria with and without prior exposure to $60 \mu\text{M H}_2\text{O}_2$.

Organism	D_{37}^a without H_2O_2	D_{37} with H_2O_2	FRF ^b
Aerobic subsurface isolates:			
UV1	7.7	6.8	1.1
UV3	10	10	1.0
Microaerophilic subsurface isolates:			
M1	1.6	2.3	0.70
M3	1.1	1.4	0.79

^a D_{37} value was defined as the UV fluence which reduced a cell population to 37% of the original number of cells. The D_{37} was calculated from the regression line of the exponential slope of the survival curve as described in Materials and Methods.

^bThe fluence reduction factor (FRF) was defined as the ratio of the D_{37} value with prior exposure to H_2O_2 to the D_{37} value without prior exposure to H_2O_2 .

TABLE 10. Intercept and slope values of the survival curves of bacteria exposed to gamma radiation. The intercept and slope were calculated from the plot of the natural logarithm of the percent survival as a function of the gamma dose.

Organism	Intercept	Slope
<u>D. radiodurans</u>	2.9	-0.01
Aerobic subsurface isolates:		
UV1	2.8	-0.33
UV2	-0.71	-0.07
UV3	0.18	-0.08
<u>E. coli</u> ATCC 25922	-0.60	-0.27
Microaerophilic subsurface isolates:		
M1	-0.02	-0.42
M2	-0.59	-0.46
M3	-0.27	-0.43

TABLE 11. D-values (krad) of bacteria exposed to gamma radiation.

Organism	D ₃₇ ^a	D ₁₀	D ₁
<u>D. radiodurans</u>	490	1,100	2,200
Aerobic subsurface isolates:			
UV1	12	27	53
UV2	14	32	65
UV3	15	34	68
<u>E. coli</u> ATCC 25922	3.7	8.4	17
Microaerophilic subsurface isolates:			
M1	2.4	5.5	11
M2	2.2	5.0	10
M3	2.3	5.4	11

^aD-values were defined as the gamma radiation dose (krads) which reduced a cell population to a specified percentage of the original number of cells. The D-values were calculated from the regression line of the exponential slope of the survival curve as described in Materials and Methods.

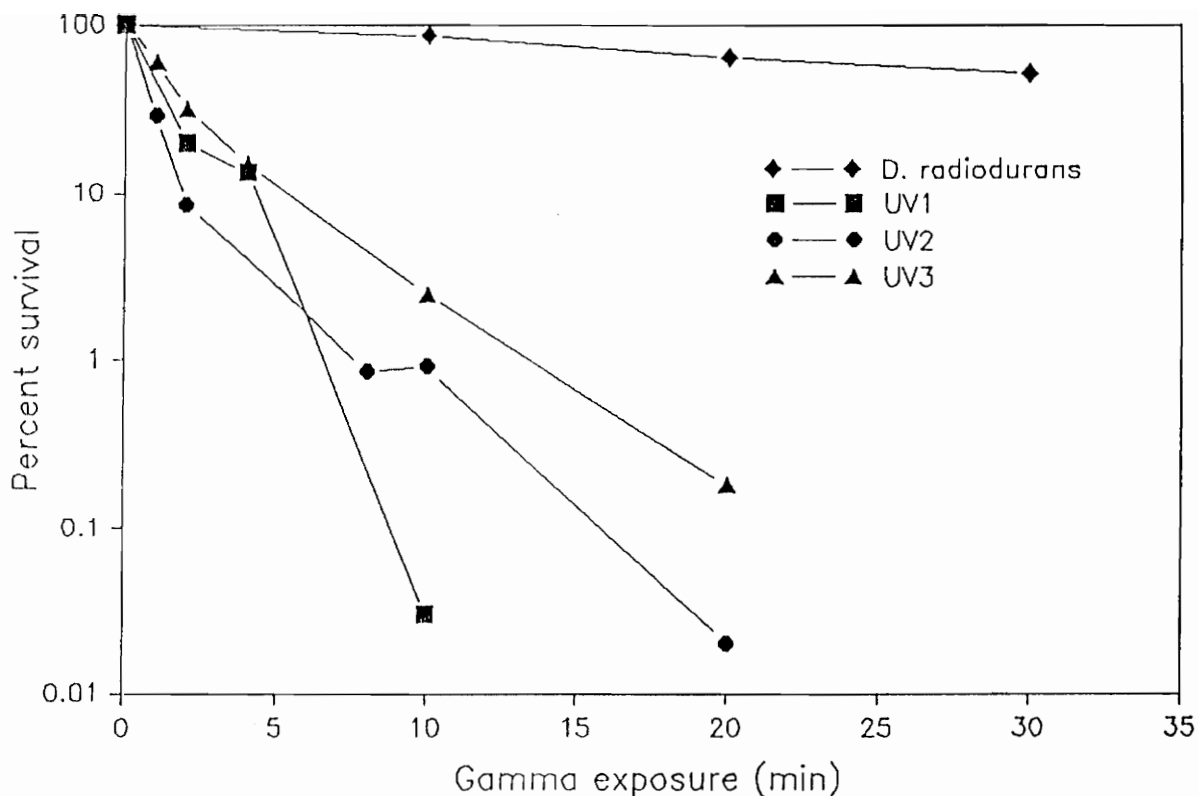


FIG. 5. Survival curves of *D. radiodurans* and 3 aerobic subsurface bacterial isolates exposed to gamma radiation. The dose rate of the ^{60}Co source was $5.8 \text{ krad} \text{ min}^{-1}$. The percent survival was plotted as a function of time of exposure.

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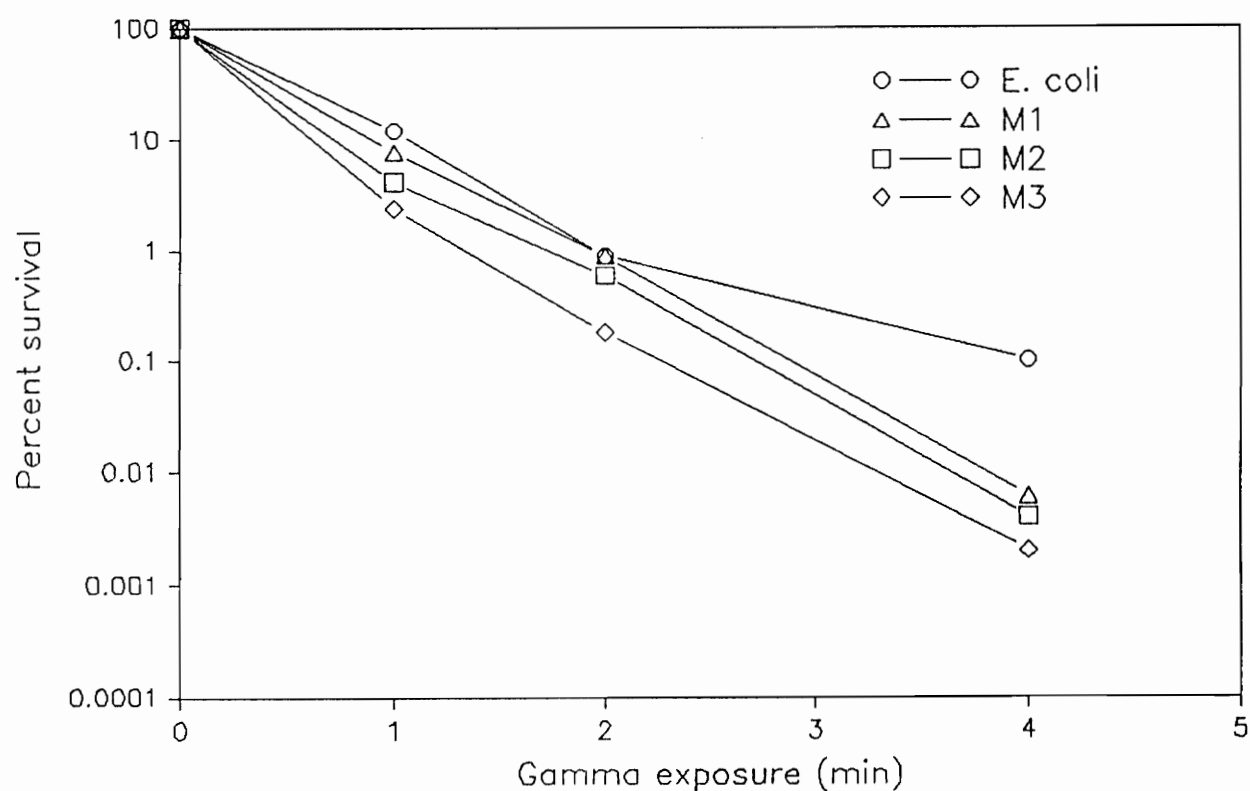


FIG. 6. Survival curves of *E. coli* ATCC 25922 and 3 microaerophilic subsurface bacterial isolates exposed to gamma radiation. The dose rate of the ^{60}Co source was 5.8 krad min^{-1} . The percent survival was plotted as a function of time of exposure.

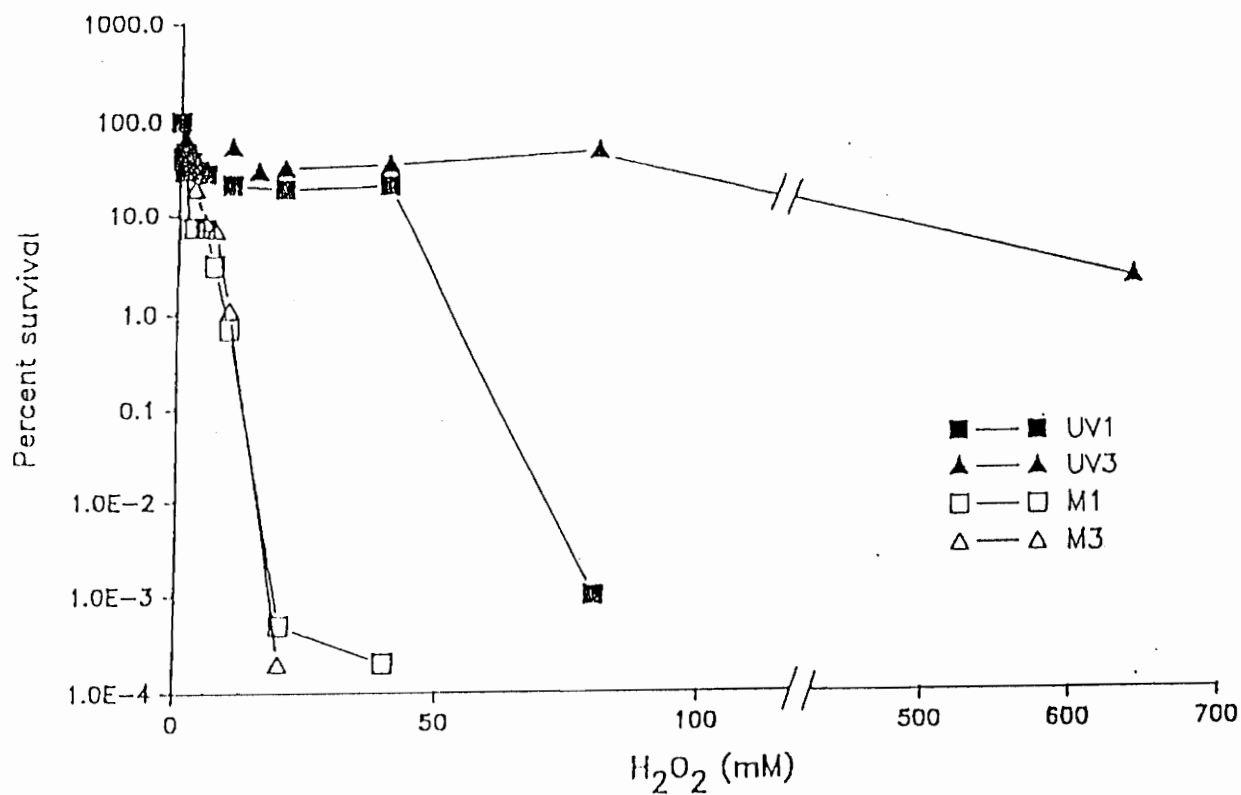


FIG. 7. Survival curves of 2 aerobic subsurface and 2 microaerophilic subsurface bacteria exposed to H_2O_2 . Cells were exposed to each H_2O_2 dose for 15 min. The percent survival was plotted as a function of the H_2O_2 dose.

TABLE 12. Intercept and slope values of the survival curves of bacteria challenged with H_2O_2 for 15 min. The intercept and slope were calculated from a plot of the natural logarithm of the percent survival as a function of H_2O_2 dose.

Organism	Intercept	Slope
<u>D. radiodurans</u>	2.5	-0.07
Aerobic subsurface isolates:		
UV1	8.4	-0.25
UV3	-0.52	-0.01
<u>E. coli</u> B	-0.46	-0.08
Microaerophilic subsurface isolates:		
M1	1.5	-0.68
M3	3.3	-0.81

Table 13. Decimal reduction values (mM H₂O₂) of bacteria exposed to hydrogen peroxide.

Organism	D ₁₀ ^a
<u>D. radiodurans</u>	110
Aerobic subsurface isolates:	
UV1	86
UV3	230
<u>E. coli</u> B	28
Microaerophilic subsurface isolates:	
M1	8.6
M3	12

^aD₁₀ value defined as the H₂O₂ dose (mM) which reduced a cell population to 10% of the original number of cells. The D₁₀ value was calculated from the regression line of the exponential slope of the survival curve as described in Materials and Methods.

5.0. DISCUSSION

5.1. UV RESISTANCE OF REFERENCE BACTERIAL STRAINS

To determine how this work related to literature reports, a range of diverse reference bacterial strains were exposed to UV light and their survival data compared to published values. Table 4 shows the previously reported D-values for a variety of bacteria and other microorganisms exposed to UV light. There is a certain amount of variation within the literature concerning UV resistance levels. For example, D. radiodurans differed within a range of 10,000 $\mu\text{Ws cm}^{-2}$. Other UV resistance ranges found in the literature include those of E. coli, Serratia marcescens, and S. aureus (Table 4).

The D_{37} of D. radiodurans obtained in this study (Table 3) was in general agreement with other reports (Table 4). This species demonstrated the highest UV resistance levels of all the reference strains tested. The order of bacterial resistance of the other reference bacteria was also similar to the literature. The four reference bacteria with the highest D-values were all gram positive. It has been hypothesized that a thicker cell wall present in some gram positive bacteria may deflect UV photons resulting in a lower dose of light actually absorbed by the organism's DNA (24,31). The presence of a shoulder region in three of the five gram positive strains at low UV doses indicated the ability to either repair the UV lesions, or to physically screen out a

portion of the radiation in that dose range. Once a threshold UV dose was reached however, an exponential rate of killing was observed in all of the bacterial strains. In fact, with the exception of D. radiodurans, the majority of reference bacteria, both gram positive and gram negative, exhibited similar survival slopes (Table 2). This result indicated a small difference in the efficiency of DNA-damage repair capabilities once the exponential portion of the survival curve was reached.

The reference bacteria used in this study exhibited lower D-values than those in the literature. These results may be due to the high fluence rate used in the study. Harm (48) demonstrated that fluence rates affected the UV survival of E. coli B and E. coli B/r. Both UV resistance and liquid-holding ability of the E. coli strains were decreased as the fluence rate was increased from a low dose rate of $0.22 \mu\text{W cm}^2 \text{ per s}$ to $80 \mu\text{W cm}^2 \text{ per s}$. The fluence rate of $700 \mu\text{W cm}^2 \text{ per s}$ in this work was much higher than the UV fluence rates normally used for bacterial UV inactivation which range from $50 - 200 \mu\text{W cm}^2 \text{ per s}$ (1,2,31). This high fluence rate may have resulted in a greater rate of DNA-damage accumulation and repair pathway saturation which manifested as an increase in sensitivity to the overall UV dose. The possession of an efficient excision repair mechanism (33) may have allowed D. radiodurans to handle the high rate of lesion formation at low dose levels while falling behind normal survival levels at the

higher doses. However the result of this high fluence rate on the other bacterial isolates was an observed increase in UV sensitivity.

It is important to identify bacteria at the strain level when comparing UV resistance since different strains of the same species may exhibit dissimilar responses to UV light (19,31) (i.e. Serratia marcescens and E. coli, Table 4). Since some of the reference bacteria differed from the strains in the literature (Table 4), it is likely that genetic differences contributed to the variation between the two groups. Many reports in the literature do not give strain identities, therefore in some cases it was not possible to determine the exact relationship between the strains in this study and those of others. Also, experimental conditions were not always described in sufficient detail to allow satisfactory comparisons of results. For example, fluence rates, UV wavelengths, growth phase of test organisms and type of sample (i.e. liquid suspension, agar plate etc.) are often not reported though all may affect the experimental results.

5.2. UV RESISTANCE OF SUBSURFACE AND SURFACE SOIL BACTERIA

Contrary to the hypothesis that subsurface bacteria have shed DNA-repair genes over geologic time in the absence of UV radiation, some of the aerobic subsurface bacteria can tolerate extreme levels of UV that are lethal to many surface bacteria. According to the hypothesis of Jagger (24), surface soil bacteria that are exposed to daily fluxes of solar

radiation should retain a high degree of UV tolerance; while bacteria whose natural habitats are screened from solar radiation should be more sensitive to UV. The data presented in this paper do not support this hypothesis. Similar numbers of UV resistant aerobic bacteria were detected from surface and subsurface soils. The majority of these UV resistant subsurface bacteria were gram positive which also agreed with the results observed from surface soil bacteria and the reference bacterial strains. No UV resistant isolates were detected among the microaerophilic bacteria. However, all of these isolates were gram negative and comparisons of survival data between 3 microaerophilic isolates (Tables 6 & 7) and the gram negative reference bacteria (Tables 2 & 3) revealed that the two groups possessed similar UV resistance levels.

There are several possible explanations for these observations: 1) Jagger's hypothesis is not valid when applied to some subsurface bacteria, that is, these organisms have conserved UV repair mechanisms although they have been shielded from solar radiation for millions of years; 2) DNA-repair mechanisms have been conserved in subsurface bacteria to protect against other DNA-damaging agents such as free radicals; or 3) the slow rate of metabolism and growth measured in these bacteria (9) have not allowed for the full course of evolution to occur compared to surface organisms.

5.2.1. Pigmentation

The role of pigmentation in DNA-damage protection is not

fully understood, however, pigments have been implicated in radiation and free radical protection (57,60). It is believed that the presence of carotenoids serve to protect against free radical-induced membrane damage rather than DNA damage. Of the reference bacterial strains tested, the two most resistant to UV were the red-pigmented D. radiodurans and yellow-pigmented M. luteus (Table 3).

The majority of UV resistant isolates from surface and subsurface soils were pigmented: 80.0% and 62.5% respectively (Table 5). The three most UV resistant subsurface bacteria (designated UV1, UV2 and UV3) possessed orange, yellow and red pigments respectively. The red pigmented isolate, UV3, was a gram positive bacterium, which resembled the morphology of the radioresistant genus Deinococcus. In addition to its color, this bacterium was a gram positive coccus which grew in pairs and tetrads. This bacterium was not as UV resistant as D. radiodurans but demonstrated resistance slightly higher than M. luteus. This isolate comprised 5-10% of the bacterial population recovered from deep subsurface soil which contained 10^6 bacteria g^{-1} soil.

The relationship between pigmentation and solar radiation has been observed (20), however, what advantage if any is gained by pigment production in the subsurface environment is not clear. One possibility is the protection of membrane components against chemically generated free radicals.

5.2.2. UV resistance and microaerophily

There was a difference in UV resistance between the two subsurface bacterial groups tested: aerobes and microaerophiles. The subsurface aerobic isolates were similar to the surface aerobes in that both UV resistant and UV sensitive isolates were present. The three aerobic isolates most resistant to UV (Table 6) exhibited initial shoulder regions which indicated the ability to successfully repair the accumulated damage from low UV doses. Exponential killing of the cells were observed only after the threshold dose for each isolate was exceeded.

In contrast to the heterogeneity of the resistance levels of aerobic subsurface bacteria to UV, all of the subsurface microaerophilic isolates were UV sensitive as defined in this study. The survival curves were linear in nature with an absence of a shoulder at the lower UV dose range. In the only reference found investigating the effects of UV on a microaerophilic organism, Butler et al (7) reported the $D_{0.1}$ of Campylobacter jejuni to be $1.80 \times 10^3 \mu\text{Ws cm}^{-2}$, indicating this bacterium was extremely UV sensitive.

In their review on the subject (28) Krieg and Hoffman defined a microaerophile as an organism that is capable of oxygen dependent growth, yet grows poorly or not at all at atmospheric levels of oxygen. Benoit and Phelps (5) described the subsurface microaerophilic bacteria as preferring oxygen levels of 1-10% and displaying only a sheen of growth at the

surface and beneath the agar of aerobically incubated plates. One possible explanation for subsurface microaerophily is that deep subsurface microaerophiles may lack efficient repair processes to tolerate oxygen-induced DNA damage, and this deficiency was reflected in the inability to tolerate high levels of UV light. However, while no UV resistant microaerophiles were detected, the level of UV resistance exhibited by the microaerophilic isolates was similar to that of the non-pigmented, gram negative reference bacteria.

5.3. PHOTOREACTIVATION IN SUBSURFACE BACTERIA

When exposed to photoreactivating light (PRL) three out of the four subsurface isolates tested demonstrated increased survival. Two of the isolates, UV3 and M3 had lower fluence reduction factors (FRF) than E. coli B which indicated a more efficient photoreactivating mechanism than the reference bacterium.

The photoreactivating enzyme or photolyase is constitutively produced in E. coli and involves the transcription and translation of one gene. The only known function of photolyase is the reversal of pyrimidine dimers when activated by near-UV or visible light (19). Due to the length of time subsurface bacteria have been screened from solar radiation, and the dedicated nature of the photoreactivation process; it was hypothesized that these bacteria would have shed the gene over geologic time and thus be unable to photoreactivate DNA damage. The data of this

experiment do not support this idea and instead favor the hypothesis that this gene has either been conserved in some subsurface bacteria for some unknown purpose; or the presence of photoreactivation in subsurface bacteria reflects a limited rate of evolution in their oligotrophic environment during the millions of years since these bacteria were deposited.

While the investigation of these two hypotheses is beyond the scope of this work, the results of studies on subsurface bacteria, including this one, indicate the latter hypothesis may be valid. Chapelle and Lovley (9) reported that the metabolic rates of subsurface bacteria were among the slowest ever recorded for any organism. Balkwill (3) and others (4,16,21,39) reported a wide range of metabolic activities possessed by subsurface bacteria although their natural environment was extremely oligotrophic. From this work, the similarity of subsurface bacterial DNA-damage resistance levels to those of the reference and surface soil bacteria indicate conservation of DNA-repair genes.

5.4. EVIDENCE OF SOS REGULATORY NETWORK

In E. coli, the SOS network can be induced by a variety of agents including radiation, H_2O_2 and chemical mutagens (32,56). Four subsurface bacteria were exposed to a sublethal dose (60 μM) of H_2O_2 for 15 min and then assayed for an increase in UV resistance. Both microaerophilic isolates demonstrated increased UV resistance (FRF < 1.0) which suggested the presence of an SOS-like process in these

bacteria. Of the two aerobic isolates, UV3 showed no change in the D_{37} value (FRF=1.0) and UV1 was slightly sensitized to UV following exposure to H_2O_2 .

It is unlikely that the aerobic subsurface isolates lack an SOS mechanism. Miller and Kokjohn (32) cite the universal presence and highly conserved nature of the recA gene in all bacteria that have been tested. It is more likely that due to the resistant nature of the aerobic isolates to H_2O_2 , the applied dose of H_2O_2 was too low to induce the SOS function.

5.5. COMPARISONS OF UV AND GAMMA SURVIVAL CURVES

Ionizing radiation resistance and UV radiation resistance in organisms are often related (13,33). Both types of radiation induce similar insults to the DNA double helix and the same major enzymatic mechanisms are utilized to repair the two types of damage. Both radiations can induce DNA strand breakage and pyrimidine dimer formation. However, while the latter is the major cause of cell lethality from UV light; strand breakage, or rather the failure to repair strand breakage, is the cause of cell death from ionizing radiation. Therefore, the survival kinetics of an organism exposed to each treatment may differ.

The large shoulder region exhibited by D. radiodurans when exposed to gamma rays was the result of the efficient excision mechanism possessed by this bacterium, which has been shown to repair lesions from both types of radiation (33,34). The exponential killing of this organism by both UV and gamma

radiation was only achieved at extreme doses compared to the survival of the other bacteria tested.

The relationship between resistance to UV and gamma radiation was also valid in the subsurface bacteria tested. The UV resistant aerobic isolates were more resistant to gamma rays than the microaerophiles as shown by the D-values in Table 9. Comparisons between the bacterial survival curves of the two radiation treatments revealed that the survival slopes were steeper when cells were exposed to UV light as compared to gamma rays. However, the calculated shoulder regions of two aerobic subsurface bacteria were smaller after gamma irradiation and absent in the case of strain UV2. This appeared to indicate that the repair mechanisms of these bacteria were not as efficient in eliminating lesions induced by low doses of ionizing radiation as with low doses of UV radiation.

The major cause of cell lethality is different for each radiation type and was described above. While UV1 was the most resistant to UV light; both UV2 and UV3 exhibited greater survival levels to gamma radiation than UV1 (Table 14). Therefore, by comparing the ranking of these isolates to UV and gamma radiation, the former two strains may be more efficient than UV1 in repairing DNA strand breakage while the opposite is true for the repair of UV-induced thymine dimers. Although the order of resistance to gamma radiation within the microaerophilic isolates was identical to that found with UV

irradiation (Table 14) there was less variation in the D_{37} values to gamma rays which indicated similar repair efficiencies for ionizing radiation among the microaerophilic strains.

5.6. COMPARISON OF H_2O_2 AND RADIATION SURVIVAL CURVES

The order of H_2O_2 resistance among the bacteria tested was also similar to that of radiation resistance. D. radiodurans and two subsurface aerobic isolates were more resistant to oxidative damage than the microaerophiles and E. coli B, which exhibited lower D_{10} values. In contrast to the results obtained with radiation, the subsurface aerobe UV3 and not D. radiodurans was the most resistant bacterium to oxidative damage (Table 11). The D_{10} value for UV3 resulted from a gradual slope which differed more than 1 log from the slope of D. radiodurans (Table 10). There was no shoulder region apparent from the calculation of the UV3 survival curve (Table 10), it is likely that the exponential portion of the curve was never reached within the range of peroxide doses used.

H_2O_2 must diffuse across the cell membrane (29) whereas UV and gamma radiation can immediately penetrate a cell's surface (31). Therefore, although the order of H_2O_2 resistance of the bacteria tested was similar to radiation resistance (Table 14), the kinetics of cell lethality may vary between the different agents. Linn and Imlay (23,29) have demonstrated a bimodal response of E. coli K12 to H_2O_2 . They have shown that mode 1 killing at low H_2O_2 doses was enhanced

in DNA-repair deficient cells while repair proficient cells only exhibited mode 2 killing at high doses. In subsurface bacteria, the presence of a bimodal pattern was not clear. In the microaerophilic strains there was a slight decrease in resistance at low H_2O_2 doses followed by an increase of resistance at intermediate doses (data not shown), however, additional testing is needed to confirm this observation.

5.7. SIMILARITIES BETWEEN ALL THREE TREATMENTS

Although the major lesion-type may be different from one agent to another, i.e. strand breaks or dimers, many of the same repair pathways are utilized. For example, the excision repair mechanism can mend both strand breaks and dimers (13,19). Exposure to both radiation and H_2O_2 induce the SOS regulatory network. While there may be variations in the shapes of the survival curves to different DNA-damaging agents, an organism resistant to one agent will likely be resistant to another. D. radiodurans was highly resistant to all three agents. Within the dose range of each treatment, this bacterium never demonstrated less than a 10% survival level. The bacterium exhibited large shoulder regions when exposed to all three challenges and only reached an exponential death rate at high doses (data not shown) compared to the survival curves of the other isolates. The rate of killing for UV and peroxide was similar with slopes of -0.05 and -0.07 respectively while resistance to gamma radiation was higher with a slope of -0.01.

The aerobic subsurface isolates, which were first isolated from the UV resistant screen, were subsequently found to also be more resistant to ionizing radiation and H_2O_2 compared to the microaerophilic isolates. They generally exhibited shouldered survival curves at low doses of each agent before reaching the exponential killing phase. There is a positive relationship between resistance to a certain agent and the demonstration of a shouldered survival curve. The shoulder indicates the dose range in which a cell population can tolerate the rate of lesion accumulation without a significant loss of survival. This tolerance is usually ascribed to the enzymatic removal of the lesion from the DNA strand with the subsequent replacement of the lost nucleotide bases (13,19,45). In contrast, the microaerophilic isolates demonstrated linear survival curves to radiation with a lack of a shoulder region. M1 and M3 did exhibit a shoulder when exposed to H_2O_2 but still possessed lower D_{10} values than the aerobic isolates to that agent. In spite of being relatively sensitive to DNA damage when compared to the aerobic isolates tested, in all three treatments, the microaerophiles demonstrated resistance levels similar to the gram negative E. coli reference strains.

5.8. SUMMARY

Some deep subsurface bacteria were highly resistant to UV light. Comparisons of subsurface bacteria to surface bacteria demonstrated similar numbers of UV resistant isolates and

corresponded to levels of UV resistance found in reference bacteria of similar gram morphology. While aerobic subsurface bacteria exhibited a range of UV tolerance from UV resistant to UV sensitive, all the microaerophilic subsurface bacteria were UV sensitive. Due to the overlap of DNA-repair mechanisms in bacteria, the occurrence of UV tolerance in subsurface isolates may reflect the conservation of enzymatic repair processes against free radical sources in the subsurface environment. However, the presence of a light-activated mechanism such as photoreactivation in subsurface bacteria favors the hypothesis that limited evolution has occurred in the subsurface environment since the sediments were deposited. Therefore, subsurface bacteria and other microorganisms may possess a physiological potential similar to that of surface microorganisms. This hypothesis is also supported by metabolic studies of subsurface bacteria that show a wide range of substrates which can be utilized by these organisms.

When exposed to other DNA-damaging agents such as gamma radiation and H_2O_2 , subsurface bacteria maintained roughly the same order of resistance as that seen with UV light. These results illustrate the feasibility of utilizing accessible DNA-damaging agents such as UV and H_2O_2 to model bacterial responses to gamma and other ionizing radiation sources.

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CURRICULUM VITA

Birth date: 7/4/64

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MEMBERSHIP IN PROFESSIONAL ORGANIZATIONS

American Society for Microbiology

American Society of Medical Technologists

CERTIFICATIONS

Medical Technologist (ASCP)

PRESENTATIONS

Survey of UV Radiation Resistance in Deep Subsurface Microorganisms. Presented December 1, 1990 at the Maryland/D.C./Virginia branch of the American Society for Microbiology.

UV Resistance of Deep Subsurface Bacteria. Abstract submitted for the annual meeting of the American Society for Microbiology, May 1991, Dallas, TX.

EMPLOYMENT

<u>Employer</u>	<u>Position</u>	<u>Dates Employed</u>
Roanoke Memorial Hospital	Medical Technologist I	7/88 - 7/89

EDUCATION

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V P I & S U	8/89 -	M.S. candidate
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V P I & S U	9/82 - 3/87	B.S.


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