An examination of constitutive direct light DNA repair and inducibility of DNA repair in two thermophilic bacteria

by Mary Ann Starkey Kirkpatrick

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology

Montana State University

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Two thermophilic bacteria, Bacillus stearothermophilus and Thermus T2 were observed for response to known DNA-damaging agents, UV radiation and the chemical mutagen, Mitomycin C. The existence of a constitutive direct light DNA repair system was discovered in Bacillus stearothermophiIus. Unlike E. coli whose dark DNA repair is UV-inducible, Thermus was not found to have a UV-inducible repair mechanism. However the presence of a DNA repair system inducible by either heat or chemicals was observed in Thermus, relating temperature-associated DNA repair with survival at high temperatures.

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Mary Ann Starkey Kirkpatrick

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11/25/85
Date

E R Vyse
Chairperson, Graduate Committee

Approved for the Major Department

11/25/85
Date

Peter F. Brussard
Head, Biology

Approved for the College of Graduate Studies

12-5-85
Date

[illegible]
Graduate Dean

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ABSTRACT

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INTRODUCTION

Biochemistry of Thermophily

Mesophilic microorgansims have a maximum growth rate at temperatures around 30-37 C; in the case of E. coli, growth ceases at 45-48 C while temperatures of 50-52 C or greater cause cell death.

Thermophilic microorganisms can thrive at temperatures up to 85 C. They are found in geothermally active areas such as hotsprings, solar-heated desert soil, as contaminants of canned food and dairy products, in hot water heaters, and in industrial effluent. Such organisms have piqued the interest of the scientific community for reasons of their proliferation at elevated temperatures and the thermostability of their macromolecules. Basic knowledge of thermophily is applicable to studies of evolution and ecology, molecular biology and biochemistry, and could lead to utilization for industrial enzymic production of marketable products (Amelunxen and Murdoch, 1977).

Temperature is only one of the variables influencing the growth of living organisms. Other environmental factors such as pH, nutrient quality and quantity, salinity, and light interact to influence the optimal and maximum growth temperatures. Under varying conditions the interrelationship of chemical structure, conformation, and function of biological molecules may vary in adapting to stress (Hochachka and Somero, 1973). Thus, attempts to explain the special ability to live

at high temperature have evolved from the observation of general physiology to a biochemical approach with concomitant examination at the molecular level.

The hypothesis that increased rates of synthesis and turnover, either by adaptation or mutation, are responsible for rapid replacement of heat-damaged proteins was advanced by Allen (1953). However, when Brock (1967) published growth rate data of various mesophilic and thermophilic bacteria at their optimum temperatures Allen's hypothesis was discounted. Brock (1967) found that thermophiles do not grow as fast at their optima as predicted by purely theoretical calculations of effect of temperature on physiology. When Ulrich (1971) combined physiological and biochemical approaches to examine a Thermus-like organism for morphological characteristics, respiratory mechanism, and regulation of enzyme synthesis, he found no major differences between mesophile and thermophile, except thermostability.

Biochemical studies of thermophiles have determined the properties of specific cell components or molecules and compared these to their counterparts in mesophiles. Fatty acids and membranes, proteins and the protein-synthesizing machinery as well as nucleic acids of several thermophiles have been isolated and examined.

Fatty acids and membranes: The membranes of thermophiles are exposed to the environment so this component of the cell was one of the first to be examined for heat stability. The fluidity of bacterial biomembranes is constantly maintained in growing cells. Variation in

complex lipid content and structural changes in fatty acid components are suggested as mechanisms to achieve the fluidity known to be important for membrane functions (Cronan, 1978). The fatty acid content of various microorganisms is known to be affected by the temperature at which they are grown (Oshima, 1978). High proportions of unsaturated acids are found at lower temperatures, while saturated fatty acids increase with increasing temperature. The presence of highly branched, longer chain, saturated fatty acids in membranes of thermophiles has been confirmed (Oshima et al., 1976). Also, a novel glycolipid constituting up to 70% of the total lipid of two strains of Thermus has been identified (Oshima and Ariga, 1976). It is conjectured that the unique lipid content of the thermophilic membrane is responsible for successful membrane function at high temperature.

Proteins: Since the primary, secondary, tertiary and quaternary structure of proteins often vary as much between proteins of the same function obtained from various mesophilic organisms as between thermophilic and mesophilic proteins of the same function, it is difficult to explain the invariable stability to denaturing conditions (chemical denaturants as well as heat) of thermophilic proteins. Various investigators have proposed that enhanced stability is due to hydrophobic (Ohta, 1966), hydrogen (Barnes and Stellwagen, 1973), or ionic (Perutz and Raidt, 1975) bonding producing conformations with larger or more densely packed protein interiors (Bull and Breese, 1973), more or different secondary sturcture (Stellwagen and Barnes, 1976), more extensively laced macromolecular surfaces (Perutz and Raidt, 1975), or

more complementary intersubunit contacts (Biesecker et al., 1977). (This topic has been reviewed by Zuber, 1976; Friedman, 1978; Amelunxen and Murdock, 1977; Singleton and Amelunxen, 1973.)

Heat-resistance is often conferred by only a few amino acid changes as shown by Merkler et al. (1981) who compared the physical characteristics of proteins of closely related mesophilic and thermophilic bacilli. Argos et al. (1979), found strategically substituted amino acids increased internal hydrophobicity and increased external polarity. Hydrophobic bonds are more stable at high temperature than at low temperature. Apparently molecular interactions within polypeptide chains are sufficient to cause thermostability.

Ribosomes: Ribosomes and the other components associated with protein synthesis are also thermostable in thermophiles. Ribosomal subunits (protein and RNA) have been found to be heat stable (Yaguchi et al., 1978). Protein elongation factors which deliver and catalyze the binding of charged t-RNAs to the ribosome are required for elongation of the polypeptide chain in protein synthesis in prokaryotes. These factors have been purified from Thermus thermophilus and compared to E. coli (Arai et al., 1978). The thermophilic elongation factors are extremely stable against heat, acid, alkali, and other protein denaturants. Thermus elongation factors showed a lack of sulfhydryl groups in contrast to those of E. coli where sulfhydryls play an essential role in catalytic function. In contrast to the monomeric forms found in the mesophile the existence of multimeric forms were

demonstrated in the thermophile.

It is apparent that no single mechanism or cell component is responsible for thermophily. As the thermostability of membranes was suggested to be due to presence of novel glycolipids as well as degree of saturation of its fatty acids and the variation in protein structure allowing function at high temperature was demonstrated, the nucleic acid portion of protein synthesis was also found to be heat stable.

Nucleic acids: The nucleic acids of thermophiles have been studied in a variety of ways including base composition; presence and action of associated polyamines; isolation and study of enzymes involved in synthesis, restriction and modification of thermophilic nucleic acids; isolation and characterization of mRNA, t-RNA, r-RNA and DNA, isolation of antibiotic resistance-carrying and cryptic plasmids, as well as the cloning of thermophilic genes with subsequent expression and characterization in mesophiles.

Heat stability of thermophilic nucleic acids increases with increased G-C content producing higher corresponding increase in melting temperature (Oshima et al., 1976). Thermophilic DNA stability may arise partially from association with divalent cations as it is known that divalent cations stabilize DNA and RNA. Thiolation of nucleic acids increases with temperature and is directly correlated with thermostability of t-RNA in the cell (Quigley and Rich, 1976).

Polyamines are generally considered to be involved in important biochemical processes such as stabilizing DNA and RNA, protein

biosyntheis, DNA and RNA biosyntheses, cell division, and acclimation to environmental stress. Novel polyamines are produced by the extreme thermophile, Thermus thermophilus (Oshima, 1975 and 1982).

Alan Malcolm has proposed in a theoretical paper (1981) that the increased G-C content of m-RNA with consequent increased stability of secondary structure should also be considered as a selection pressure in the thermophilic environment and that the most common amino acid changes between mesophiles and thermophiles recorded by Argos et al. (1979) are consistent with this hypothesis. Single base changes found in the m-RNA codons of thermophiles either increase the stability of secondary structure or have little effect (none would decrease secondary structure).

Stenish and Madison (1979) compared the stability of m-RNA in mesophiles and thermophiles, found the half-life of m-RNA to decrease as growth temperature increased but discovered the "stability index" (half-life of mRNA/doubling time of cells) to be constant for each organism regardless of temperature. This supports the concept that kinetic considerations play a significant role in thermophily; the half-life of the m-RNA is a fixed fraction of the doubling time.

Nucleic acids of thermophiles and mesophiles have been shown to vary in nucleotide content, presence of novel polyamines, structure of enzymes involved in synthesis, and restriction and modification enzymes. However, there are many similarities in basic organization of genetic material and expression of information. Thermophilic DNA and RNA polymerases (Kaledin et al., 1980; Chien et al., 1976; Date,

1975), DNA methylase (Sato et al., 1980), and DNA restriction enzymes (Sato et al., 1977) have been isolated and characterized. The properties demonstrated by these enzymes are similar to those of other thermostable proteins. This is also true of enzymes produced from genes cloned from thermophiles (Nagahari et al., 1980). A circular dichroism study of the complex between promoter DNA and Thermus RNA polymerase (Tsuji, 1980) showed only more melting in the promoter region than found in E. coli, confirming the similarities of the two organisms.

Research on DNA repair and mutagenesis in mesophilic bacteria, initial observations of the filamentous highly thermophilic bacterium, Thermus, and recent reports that a protective response to heat stress can be induced in mesophilic prokaryotes and eukaryotes by agents that can induce changes in DNA repair activity associated with filamentation suggested to Dr. Guylyn Warren a possible natural association between growth at high temperature, DNA damage and repair.

DNA Damage and Repair

DNA Damage: Four main types of DNA alterations or damage have been studied: 1) Dimerization of two adjacent pyrimidines on the same DNA strand when the pyrimidines become connected by a cyclobutane ring, 2) chemical alteration of bases by deamination or aklylation, 3) introduction of covalent crosslinks between bases on two strands, and 4) breaks in one or both strands. Any of these damages can result in lethality or an altered coding property or mutation.

Mutations can be spontaneous, possibly arising from enzymatic

dysfunction during DNA replication or recombination. Mutation, in a broad sense, although a heritable change, may not affect the phenotype or be recognized. However, the term will be used in this manuscript to refer to a heritable change in nucleotide sequence of an organism which is recognized by its effect on the phenotype of the organism.

A mutagen is an agent which causes changes, as described above, in genomic nucleic acid and increases the mutation rate above the spontaneous level as observed phenotypically. Known mutagens present in the environment include radiation (UV and X-rays), chemical mutagens (alkylating and deaminating agents, base analogs, intercalating agents, and cross-linking agents) and transposons.

1. Radiation.

a) Ultraviolet (UV) light causes formation of dimers between pyrimidine bases on the same strand (intrastrand) of DNA. The pyrimidines become connected by a four carbon cyclobutane ring. (See diagram and detail in UV section below.)

b) X-rays cause breaks in the phosphodiester backbone in one or both strands of DNA.

2. Chemical mutagens effect modifications in DNA bases in situ by deamination, alkylation, or the addition of a variety of bulky adducts. Chemical mutagens also include: structural analogs which vary in bonding with the partner base; intercalating agents which insert during replication, distort the base pairing and leave after replication resulting in a gap or an added base in the newly synthesized strand; and cross-linking agents which form interstrand cross-

links posing an absolute block to replication and transcription.

3. Transposons, or units of DNA that have the capability of moving from one DNA moleule to another, result in rearrangements and deletions in the molecule that was left and insertion and disturbance of DNA coiling in the molecule entered.

DNA Damage - UV and MC: UV and Mitomycin C were chosen for examination of the thermophiles' response to mutagens. The pyrimidine dimer caused by UV is the best researched lesion. The cross-linking mechanism of Mitomycin C provided a second mechanism of DNA damage for observation.

UV Damage: DNA efficiently absorbs light in the range of 240-300 nm resulting in excited energy states of the bases and causing a variety of photochemical reactions (Wang, 1976). The principle product, pyrimidine dimers, causing the principle biological effects, lethality and mutagenisis, is formed when two adjacent pyrimidine bases on a strand are linked together by a four-carbon ring (Fig. 1). The two bases are pulled out of alignment, the hydrogen bonds to complementary bases are broken, and the DNA backbone is distorted, preventing the correct pairing of the two bases on each side of the dimer. The presence of a single dimer can interrupt transcription or replication. Even if replication resumes on the other side of the dimer, a gap is left in the newly synthesized strand, blocking transcription of the entire transcription unit and aborting replication in the next cycle (Hanawalt et al., 1979).

Mitomycin C (Fig. 2) is metabolically reduced by a quinone reductase in the cell to a hydroquinone derivative which alkylates and extensively cross-links DNA (Iyer and Szybalski, 1963). The biological significance of interstrand cross-linking is evident from studies on transforming DNA and bacterial viruses (Kohn et al., 1963; Becker et al., 1964)). One interstrand cross-link is sufficient to cause the inactivation of at least 3,000 base pairs within a DNA molecule, presumably as a consequence of blocking complete strand separation for replication. E. coli mutants defective in one or more uvr genes (excision repair) are more sensitive to Mitomycin C than wild type strains. While simple alkylation damage is not repaired by excision repair, cross-linking caused by the bifunctional Mitomycin C requires excision repair (Fishbein et al., 1970; Cole et al., 1976).

DNA Repair: For every organism, life and continuity from generation to generation depend on the long-term stability of its hereditary material, the DNA. Since all cells are sensitive to damage by radiation and chemical agents in the environment, a system of removal of lesions and restoration of the intact DNA appears to have been adopted. It is not possible for DNA polymerase III to replicate areas of DNA containing dimers or cross-links, although it can restart after the damaged region has been passed. Howard-Flanders (1975) has shown that daughter DNA molecules replicated from UV-damaged DNA contain gaps approximately the size of one or more Okazaki fragment indicating

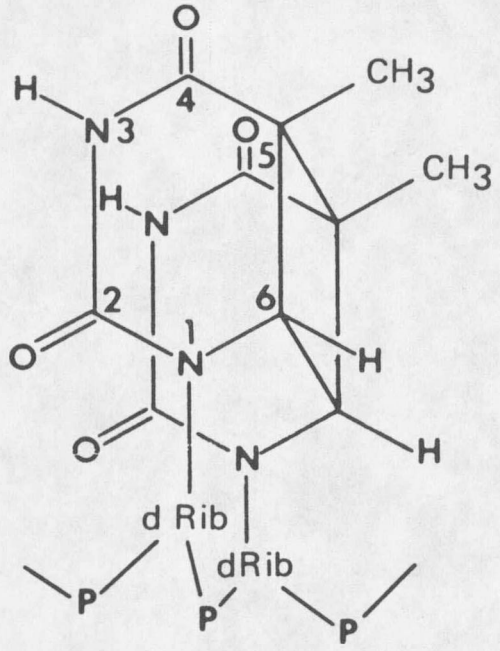


Figure 1. Structure of a thymine dimer resulting from ultraviolet irradiation of DNA (based on Davis, B.D., Dulbecco, R., Eisen, H.N., Ginsberg, H.S. Microbiology, Third Edition, Harper and Row, Maryland, 1980).

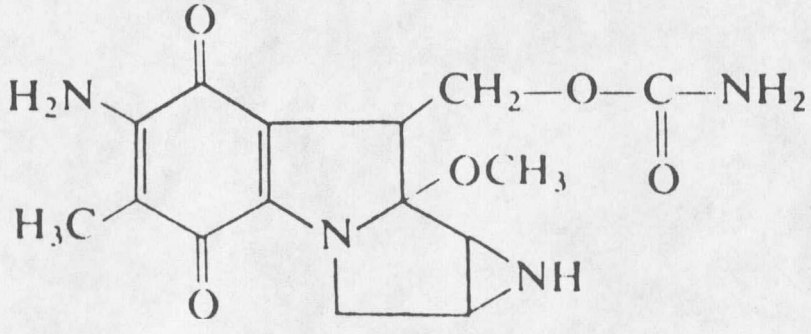


Figure 2. Structure of Mitomycin C (based on Fishbein, L., W.G. Flamm, and H.L. Falk, eds., Chemical Mutagens. Academic Press, N.Y., 1970).

resumption of replication does occur with concomitant gaps in the daughter strand.

DNA repair involves recognition of a lesion by a protein that can initiate the biochemical reactions which lead to elimination or circumvention of the lesion. While specific enzymes repair some specific base modifications or correct a chemical alteration, other types of repair are more general in nature and can either repair a variety of lesions or allow a second chance at specific repair through recombinational processes. The major types of DNA repair known in bacteria are photoreactivation, base substitution, excision repair, the adaptive response and recombinational or post-replicational repair. Except for photoreactivation, specific proof-reading functions in DNA replication and excision repair, DNA repair is largely error-prone.

1. Photoreactivation.

Photoreactivation was discovered as a reduction in the lethal effect of far-UV irradiation by a subsequent exposure to longer wavelengths. It has been demonstrated in many cellular systems including bacterial and human and acts on pyrimidine dimers only. The photoreactivating enzyme binds to the dimer-containing region of the DNA thus generating a DNA-enzyme chromophore that absorbs visible light to catalyze cleavage of the joined bases without breaking any phosphodiester bonds.

Photomediated recovery from UV damage was discovered in bacteria and bacteriophage in 1949 (Kelner; Dulbecco). The mechanism was

characterized in 1962 (Rupert). Recently, an alternate role for the photoreactivating enzyme was suggested by Yamamoto et al. (1983) who discovered an *E. coli* rec A mutant to be less sensitive to UV in the presence of a photoreactivating enzyme in the dark. Photoreactivation, however, can be masked by an efficient excision repair system.

2. Excision Repair.

Different modes of excision repair of damaged DNA have been discovered in different organisms. In *E. coli* three distinct loci are known to be required for excision of damage in UV-irradiated DNA. The *ultra violet light damage repair*, or *uvr*, genes are responsible for three proteins that associate (Nakabeppu and Sekiguchi, 1981) to make the UVRABC enzyme, now cloned and characterized by Sancar and Rupp (1983). UVRABC is responsible, in the presence of Mg^{++} and ATP, for making two cuts, one on each side of the damaged DNA, thus removing a 12-13 nucleotide long, single-stranded fragment of DNA. The gap is then filled through action of DNA polymerase I and sealed by DNA ligase. It is suggested that the enzyme may bind to the relatively unstable section of the 12-13 base pair fragment resulting from DNA damage, the instability enhanced by binding of UVRA and UVRC to single stranded DNA (Seeberg and Steimem, 1982; Sancar and Rupp, 1979) and that the DNA polymerase I exonuclease activity may enhance removal of the excised strand. The cuts, displaced from the sites of damage, can repair a broad spectrum of damages without precise recognition of particular adducts. The enzyme acts on DNA which has been treated with UV-irradiation, Pt (II) compounds, psoralen plus near-

ultraviolet, nitrous acid or Mitomycin C (Brash and Haseltine, 1982).

Kenyon and Walker (1981) have suggested that the UVRA and UVRB proteins are produced at constitutively low levels but induced to higher levels of production by the control elements of the inducible "SOS" response of bacteria activated when repair has not occurred and DNA replication is blocked at the site of damage.

3. Postreplicational repair.

Postreplicational repair takes advantage of Okazaki-sized gaps which are left by polymerase III in the newly synthesized DNA opposite the damaged region. Postreplicational repair, then, is a system of retrieval, whereby material from one strand of a duplex of DNA can be used to repair the gap in another. The mechanism of crossovers between two daughter molecules, whose gaps do not usually coincide is recognizable by electron microscopy of DNA (Rupp and Howard-Flanders, 1968); Rupp and coworkers (1971) demonstrated that discontinuities were formed in DNA in an excision-defective strain. The lesion remains on one strand; however, another attempt to replicate can be made and may be successful with replication of the new recombinant molecule. The daughter strand gap repair requires a functional reccombination rec A gene as well as polymerase, pol A or pol C.

4. The Adaptive Response.

When exposed to low concentrations of methylating or ethylating agents, E. coli becomes resistant to the mutagenic and lethal effects of higher doses of the same agents (Jeggo et al, 1977 and 1978). The independently regulated pathway was first discovered by Samson and

Cairns (1977) and has been termed the adaptive response (Jeggo et al., 1977). The inducing signal for the network is unknown. The positively acting regulatory element of the adaptive, ada, locus is suggested by Walker (1984) to be specifically induced by the adduct of the O^6 alkylation of guanine. The ada locus consists of an operon coding for two proteins (Lindahl, 1982). Also known to be involved in the adaptive response is the unidentified locus coding for O^6-alkylguanine-DNA alkyltransferase (Lindahl et al, 1982) (which catalyzes the transfer of the methyl or ethyl group from the alkylated guanine to the protein itself), a protein of broad specificity, the 3-methyl adenine-DNA glycosylase II which is the product of the alk A gene (Evenson, G. and E. Seeberg, 1982) and an unknown number of other genes.

Although methylating and alkylating agents can introduce lesions that mispair and result in mutation, other lesions caused by these agents induce another mode, "SOS" repair, which results in active mutagenesis.

5. SOS Repair.

The term "SOS functions" has been designated for a complex group of responses in E. coli that appear to be coordinately regulated. Included in these responses are inhibition of cell division, filamentous growth in repair-deficient mutants associated with induction of SOS repair, inhibition of postirradiation DNA degradation, induced bacterial mutagenesis, induction of prophage, as well as Weigle reactivation and Weigle mutagenesis. The latter two responses

were discovered by Weigle (1953) when he observed that UV-irradiated bacteriophage yielded more plaques and a higher proportion of mutants when plated on lightly UV-irradiated E. coli than when plated on unirradiated cells. The same genetic and physiological requirements applied to prophage induction (Defais et al., 1971) which requires site specific recombination. The mutagenic response operated on host DNA as well. These observations led to the proposal that the regulated functions represent an inducible response of bacteria to unrepaired damage in their DNA or an "SOS" signal (Radman, 1975).

Induction of the SOS responses is accompanied by the appearance of a prominent 40 Kd protein (Inouye, 1971) now known to be (constitutively produced in low levels) the recA gene product. Synthesis of the recA protein is regulated by the gene product of lexA, a repressor of recA and a number of other genes, and by the recA protein in the active form, a protease or a cleavage stimulator (George et al., 1975). DNA degradation at incision sites as well as at stalled replication forks may result in induction of the SOS response. The signal may be a complex of single-stranded DNA, a single-strand-binding protein, plus an oligonucleotide (Oishi, 1978). Witkin (1974, 1975, 1976) suggested that SOS could operate as (1) an error-prone variant of recombinational repair or (2) as a nonrecombinational repair system which polymerizes DNA past the pyrimidine dimer or other noncoding lesions in the template strand. Cooper and Hanawalt (1972a,b) presented biochemical evidence that there are two types of repair which function in closure of excision gaps, one of which is dependent

on rec gene products. The evidence supports Witkin's proposal.

The principal model for Weigle reactivation and mutagenesis proposes "transdimer synthesis" (Clark and Volkert, 1978) as a result of modification of normal DNA polymerases (perhaps the 3'--5' editing exonuclease activity) by an inducible protein thus facilitating replication past lesions and increasing probability of error. In cases of massive damage with resultant closely-spaced lesions on opposite strands, excision and resynthesis initiated at one lesion would stop at a second closely-spaced lesion on the opposite strand and would require transdimer synthesis for completion resulting in a long repair patch and production of mutations.

Mutation in E. coli is dependent upon the gene products of the recA$^+$ and lexA$^+$ genotype and the function of other genes, described in the next section. Mutation is mediated by all the pathways described for SOS repair:

a) transdimer synthesis due to inducible suppressed 3'--5' editing activity of polymerases;

b) daughter strand gap repair by sister strand exchange;

c) excision repair plus transdimer synthesis in cases of massive damage resulting in long patch repair.

Mutation rates are decreased in the rec A$^-$ and lex A$^-$ strains.

6. SOS Repair-Deficient Mutants of Escherichia coli.

Genetic analysis of repair mutants has revealed a major portion of what is known about the molecular basis of SOS repair and mutagenesis. Many of the mutants studied were isolated for other traits and

subsequently identified as affecting mutagenesis.

a. Alleles of rec A (recombination-deficient) and lex A showed rec A and lex A to be required for SOS and mutagenesis, to be controlling a variety of physiological responses and suggested the system had to be induced (Walker, 1984).

b. Temperature-induced filamentation, or tif mutants, show the SOS inducible responses to be inducible by temperature without DNA damage, with concomitant high levels of rec A protein and subsequent "activation" of it to the protease which acted on repressors of the variety of SOS functions described earlier (Gudas and Mount, 1977).

c. When non-mutable (defective in SOS) mutants were screened for and identified, mutants other than of the induction process itself were identified. In this way umu C and umu D mutants were discovered (Kato and Shinoura, 1977). Umu mutants are still capable of expressing a variety of SOS responses and therefore code for products required for SOS processing and not essential to the cell. The umu C and D loci have now been cloned (Elledge and Walker, 1983). Characterization of the activity of the gene product so far confirms the inferences from earlier work with mutants.

d. Strains carrying the single strand DNA binding (ssb) mutation are also deficient in SOS processes. The ssb gene product which binds strongly to single-stranded DNA is apparently playing a positive controlling role in the system (Myer et al., 1979).

e. The long form mutation (lon) allows filamentation in E. coli cells. The lon gene product is an ATP-dependent protease which

regulates the amount of filamentation by affecting the rate of degradation of the suppressor of lon or sul gene product (Charette et al., 1981; Chung and Goldberg, 1981). Lon^+ cells filament transiently; lon^- cells filament indefinitely and are sensitive to UV. The sul A protein can be induced to inhibit filamentation in lon^+ cells; sul A is SOS-induced. The lon gene product also affects the degradation of a mutant sigma subunit of RNA polymerase. Since a high temperature protein (htp) mutant is similar, slowing degradation of the sigma subunit of RNA polymerase; (Walker, 1984) suggests that the htp R may be involved in controlling the activity of the lon protease and other proteases.

7. Heat Shock.

Another inducible protective measure for cells is the "heat shock" protective response against thermal killing. In E. coli a shift up in temperature elicits changes in the production of most cellular proteins, some cease being made, some are transiently induced (Yamamori et al., 1978). The original discovery of heat shock phenomena was the heat-induced puffing of Drosophila polytene chromosomes (Ritosa, 1962). "Puffing" is indicative of active gene loci in the polytene chromosomes. Subsequently an analogous response to heat was found in many other species including bacteria, mammalian cells, and plants (Schlesinger, 1982). In E. coli a group of 13 heat-induced proteins which have been found by Neidhardt et al. (1981, 1982, 1983) to constitute a High Temperature Regulon (HTR) which is dependent upon a positive regulatory protein. By 1985 a total of 17

heat shock responsive proteins had been found (Neidhardt et al, 1984).

The heat shock response is a ubiquitous response to stress (Hightower, 1980). A variety of agents can induce changes in gene activity similar to those caused by heat shock. Drosophila cells show a significant increase in the synthesis of three small heat shock proteins with eight of ten teratogen treatments while seven drugs that do not inhibit differentiation do not induce heat shock proteins (Buzin et al., 1982). Also, in Drosophila, amino acid analogs, sulfhydryl-reacting reagents, transition metal ions, uncouplers of oxidative phosphoxylation, viral infection, ethanol, a variety of antibiotics, some chelators and ionophores induce the response. Krueger and Walker (1984) found that heat shock proteins in E. coli are induced by SOS repair-inducing agents, UV and naladixic acid. Induction was found to be controlled by the high temperature protein (htp R) gene product, a positively acting element required for expression of heat shock genes in E. coli (Neihardt and Van Bogelen, 1981). So, UV and naladixic acid induce the SOS system and the independent regulatory system, the heat shock response. The nature of the inducing signal is still unknown. The functional significance of the heat shock response is also unknown. However, there is evidence that DNA-associated proteins in E. coli are clearly altered with heat induction (Pellon et al, 1980, 1981, 1982). Heat shock proteins, then, could be required for the stability of chromosome structure or involved in repair of heat damaged DNA. Walker (1984) speculates that the htp R-controlled gene products are involved in degradation of SOS-

induced proteins which would be deleterious to the cell if they persisted after completion of the SOS response.

8. The bacteria used for research work are the intermediate thermophile, Bacillus stearothermophilus, and the extreme thermophile, Thermus (strain T2).

Bacillus stearothermophilus (Gordon, 1923) is a gram-variable, motile, straight rod-shaped organism which is capable of growth at 65-70C with optimum growth at 55C; and which produces heat-resistant endospores. Spores are formed in soil in all climatic zones. Vegetative growth is rapid in many foods of pH above 5.0 (if held at an appropriate elevated temperature), in heating compost, and in contaminated, improperly processed, canned foods. B. stearothermophilus has been often used in laboratory research of thermophily.

Thermus aquaticus, a non-motile obligate aerobe which is a gram-negative rod resembling E. coli, was first described by Brock and Freeze (1969) who reported their initial isolates as filamentous, commonly finding filaments in 65-70C and stationary phase cultures. Electron micrograph studies of extremely thermophilic bacteria show Thermus, in contrast to E. coli, to have a regular scallop-like connection to the inner membrane/cell wall giving isolates an annelid-like appearance (Ramaly et al., 1978). The genus has appeared in hot water heaters (Brock and Boylen, 1973) and naturally-occurring water that has been contaminated by thermal effluent (Degryse et al., 1978).

9. Statement of Research Problems.

The purpose of the research reported here was to combine current

knowledge of the fields of thermophily and DNA repair in a study of two thermophilic microorganisms in order to advance knowledge in the area of survival of life at high temperature. The study included:

a. Identification and differentiation of one thermophilic strain by morphological examination and biochemical testing.

b. Development of appropriate medium for study of the two thermophiles at 55C and 70C.

c. Exposure of Bacillus stearothermophilus and Thermus T2 to the known DNA-damaging agents, UV and Mitomycin C, for determination of their survival patterns.

d. Examination of the two strains for the existence of constitutive photoreactivation repair systems similar to their mesophilic counterparts.

e. Determination of the existence of an inducible repair system (SOS) in the two organisms.

f. Examination of the relationship of heat-tolerance to inducible repair and filamentation in Thermus.

MATERIALS AND METHODS

Source of organisms

1. Thermus was originally isolated by Ulrich from an alkaline thermal spring in Yellowstone Park. Strain T2 is a mutant laboratory strain which is non-mucoid and consequently easier to work with in the laboratory. The culture used for these studies was obtained from the American Type Culture Collection (ATCC #27737).

2. The strain of Bacillus stearothermophilus used in this study was originally isolated as a contaminant in the laboratory of Dr. Gordon Julian of the Montana State University Biochemistry Department and was identified during the course of this study.

3. Wild-type E. coli used in photoreactivation experiments was strain H53 isolated from chicken caecum in the laboratory of Dr. David Sands (Plant Pathology Department, MSU).

4. Control E. coli for UV survival experiments was strain AB1157 λ-(K12) (Bachman, 1972).

Identification and Differentiation of Bacillus

1. Staining procedures. Gram stain and Malachite Green spore stain were performed according to Conn's Biological Stains (Lillie, 1936).

2. Sporulating agar, obtained from Dr. Nels Nelson, Department of Microbiology, Montana State University, was prepared according to Gordon et al. (1973), inoculated with a loop and incubated at 55 C for

18 hours.

3. Reduction of nitrate and nitrite. Nitrate reduction medium was obtained from Dr. Nels Nelson, Department of Microbiology, Montana State University, and was prepared according to Gordon (1973). Medium to detect production of N_2 gas was prepared by adding a trace of Zn to the nitrate reduction medium and placing it in an Inverttube which, during autoclaving procedure, expells the gas and fills the inverted tube with liquid, thereby allowing any gas production by the organism to be observed as bubbles in the inverted tube.

Bacterial Growth Conditions

1. Media

A. The routine studies of Bacillus employed a medium containing 0.5% Tryptone (Difco) and 0.5% yeast extract in a basal salts solution (10 ml each of Solutions I, II, III per liter). The pH was adjusted to 7.7 preautoclaving which shifted to a final pH of 7.5 during the autoclave procedure.

Solution I	$(NH_4)_2SO_4$	(3.96 g/l)
	KCl	(7.46 g/l)
	NaCl	(23.33 g/l)
	$Na_2HPO_4.2H_2O$	(46.27 g/l)
Solution II	$Ca(NO_3)_2.4H_2O$	(1.18 g/l)
Solution III	$MgSO_4.7H_2O$	(9.8 g/l)
	or	
	$MgSO_4$	(4.8 g/l)

The agar content (Difco, Microbiological grade) of solid medium was 2%

(20 gm/l). Plates were dried at room temperature for several days or at 70°C for 1 1/2 hours before use.

B. Media used for the culture of Thermus were: 1) the basal salts plus yeast extract and tryptone, pH 7.8, described by Ulrich (1971); or 2) a simpler medium worked out by Dr. Emmet Johnson at Tulane University (personal communication) specifically for the culture of Thermus.

Johnson's Thermus medium:

Salts Solution I (100X)

$(NH_4)_2SO_4$	4.0 g
KCL	7.0 g
$Ca(NO_3)_2 \cdot H_2O$	1.2 g

brought to 1,000 ml with water

Salts Solution II (100X)

NaCl	20 g
$Na_2HPO_4 \cdot 7\ H_2O$	30 g
$MgSO_4 \cdot 7\ H_2O$	10 g

brought to 1,000 ml with water

Thermus medium

Salts Solution I	10 ml
Salts Solution II	10 ml
$FeSO_4$ (0.05%)	1 ml
Yeast extract	2 g
Difco tryptone	2 g

added to 1,000 ml water to prevent precipitation of salts.

Two gelling agents were used in conjunction with the two media; 2% Bacto-agar or 0.8% Gelrite were compared in each recipe. When Gelrite was used, 1 g $CaCl_2 \cdot 2\ H_2O$ was dissolved in one liter of water followed by 8 g Gelrite and finally the other ingredients. The magnesium content of Johnson's medium was also increased to 1 g $MgSO_4 \cdot 7\ H_2O$ per liter for use of Gelrite. Finally, the pH was adjusted with NaOH to a preautoclave pH of 8.1 which resulted in the final pH 7.8 after autoclaving.

Thermus X-Gal Medium

$CaCl_2 \cdot 2_2O$	0.5 gm
$MgSO_4 \cdot 1\ H_2O$	0.5 gm

in 1 liter of water. Then add

Gel-rite	4.0 gm

followed by

Johnson's Solution I	5.0 ml
II	5.0 ml
Casamino acids	5.0 gm
Vitamins (biotin, lipoic acid, B_{12}, para aminobenzoic acid)	1.0 ml each

Adjust pH to 8.1 (about 0.7 ml 4M NaOH).

Autoclave before adding the following:

Lactose (10%)	10.0 ml
X-Gal	20.0 mg (dissolved in dimethyl formamide)
IPTG	60.0 mg

2. Incubation.

Covered shaking water baths at the appropriate temperatures were set to rotate or shake at 100 rpm for liquid cultures. Cultures on solid media were incubated at: a) 58 C in a Hotpack humidified incubator; twenty-four hours for Bacillus and 3-4 days for Thermus were required for growth; b) 70 C in sealed plastic Petri dish bags with a blank plate as a spacer and 5 ml sterile distilled water placed in bag bottom to prevent dehydration; 2-3 days were required for growth of Thermus.

3. Buffers

Bacterial washes and 10-fold dilutions of bacterial cultures were made in appropriate liquid medium or the following phosphate buffers:

a) Bacillus	NaCl	8.5 gm	
	K_2HPO_4	5.7 gm	pH = 7.2
	KH_2PO_4	3.4 gm	
b) Thermus	NaCl	8.5 gm	
	K_2HPO_4		pH = 7.8

Microscopy

All light microscopic observations and photographs were made with a Wild (Heerbrug) M-20 microscope fitted with a 3.5 mm camera attachment. Kodak high contrast copy film was used and developed with Kodak developer D-19.

Electron microscope examinations required bacteria grown in standard growth medium to be washed in phosphate buffer. Either a

drop of this suspension or one made of a colony from solid medium suspended in water was placed on a 300 mesh copper grid coated with 0.2% Formvar in chloroform.

Platinum shadow casting was done with platinum-paladium wire. Observations were made with a Zeiss (EM 952) electron microscope and associated camera.

DNA Damaging Treatments

1. Exposure to UV. The Sylvania GTE 8 watt germicidal lamp used for ultraviolet irradiation generated a dosage of 190 u watts/cm^2 as measured by a Spectroline DRC-100x digital radiometer.

A. Cultures on solid media were irradiated according to Greenberg (1967).

B. Liquid Cultures. At a density of approximately 10^8 cells per ml, two ml aliquots of washed cells suspended in the appropriate phosphate buffer were placed in disposable 60 x 15 Petri dishes and treated with UV as above. The cells could then be diluted and plated directly or used for a photoreactivation treatment.

2. Exposure to Mitomycin C (MC). Test of susceptibility of Thermus to MC was achieved by plating a suspension of the organism on solid medium and incubating at the appropriate temperature (58 C or 70 C) for approximately 24 hours before addition of sterile filter discs upon which were placed varying concentrations of the drug. After another one to two days of growth the zone of inhibition surrounding the disc was measured.

3. Repair assays.

Direct Light Repair. Photoreactivation experiments were variations in the protocol for teaching laboratory experiments with E. coli (Seeley and Van Demark, 1981). Cells in log phase were washed in buffer of pH appropriate for each organism then resuspended in buffer at 1/10 volume. Two milliter aliquots were placed in sterile disposable 60 x 15 Petri dishes and irradiated for varying times. The plates were immediately placed in plastic bins and covered with foil to prevent repair due to overhead lights. As soon as possible the cells were transferred to sterile tubes and placed in a constant temperature bath to prevent overheating from the lightbulb used for repair. The bulbs used were either white 250 watt Sylvania BBAS or blue 250 watt Sylvania BCA placed in a photoreflector shade six inches above the tubes. Controls included:

Non-irriated cells:

Treatment #	
1	Constant temperature bath; dark (foil-covered)
2	Constant temperature bath; light
3	Room temperature bath; dark

and irradiated cells:

Treatment #	
4	Constant temperature bath; dark
5	Room temperature bath; dark
6	Constant temperature bath; light

Percent recovery was calculated by the folowing equation:

$$\% \text{ recovery} = \frac{\#\ \text{Survivors (Treatment \#6)}}{\text{Total \#\ (Treatment \#1)}}$$

Survival

Dilutions of appropriate cultures were plated and then irradiated for various lengths of time. Colony counting was done with a New Brunswick Biotran II automatic colony counter. Spectrophotometric determination of cell density in broth was made using LKB ultrospec 4050 at a wavelength of 550 mu. Survival rate was calculated as number of cells surviving irradiation (irradiated plate count) divided by number of cells irradiated (plate count, no irradiation).

RESULTS

A. The Organisms of the Study

1. Identification of Bacillus stearothermophilus

Initial observations. The culture obtained from the Montana State University Biochemistry Department and initially thought to be Thermus deviated from normal Thermus behavior in generation time, optimum pH, and growth temperature. No growth on minimal lactose medium indicated a lack of a functioning lac operon. Gram staining was not conclusive as cells from a colony on solid media appeared as Gram negative, elongated rods. Further investigation, however, showed the organism to be a spore-former with flagella, not filamentous in liquid culture at 55 C or 70 C.

Morphology. Gram stains of fresh cultures showed the bacterium to be Gram-variable short rods (Plate 1). When grown on sporulating agar for 18 hours it produced many spores which, when stained with Malachite Green, were easily detected under the light microscope. The spores were eliptical in shape and located in the terminal region of the cell. Shadow casted specimens observed under the electron microscope indicated eliptical terminal spores (Plate 2) and flagella (Plate 3).

Physiological Characteristics. Optimum pH for growth on basal salts plus Tryptone and yeast extract is 7.5. Optimum temperature for growth is 55 C. Generation time (doubling time) under these condi-

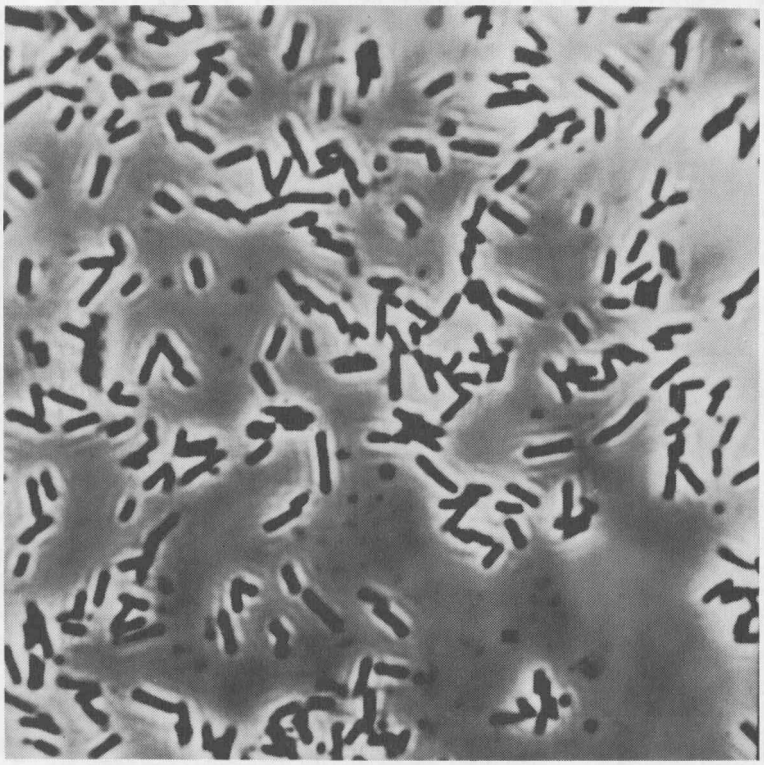


Plate 1. Bacillus (Gramstain) 50X

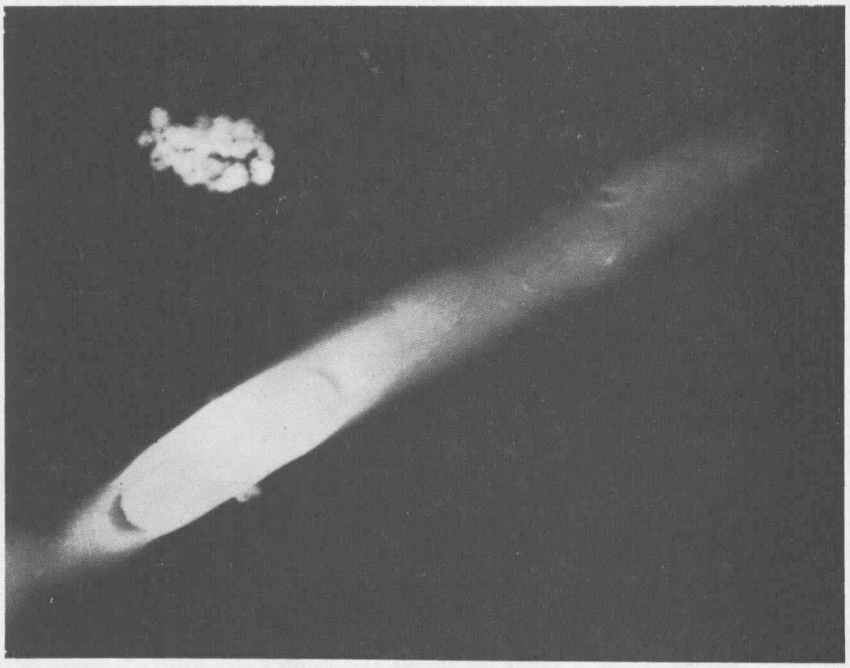


Plate 2. Bacillus terminal elliptical spore.
Electron micrograph 31,000X

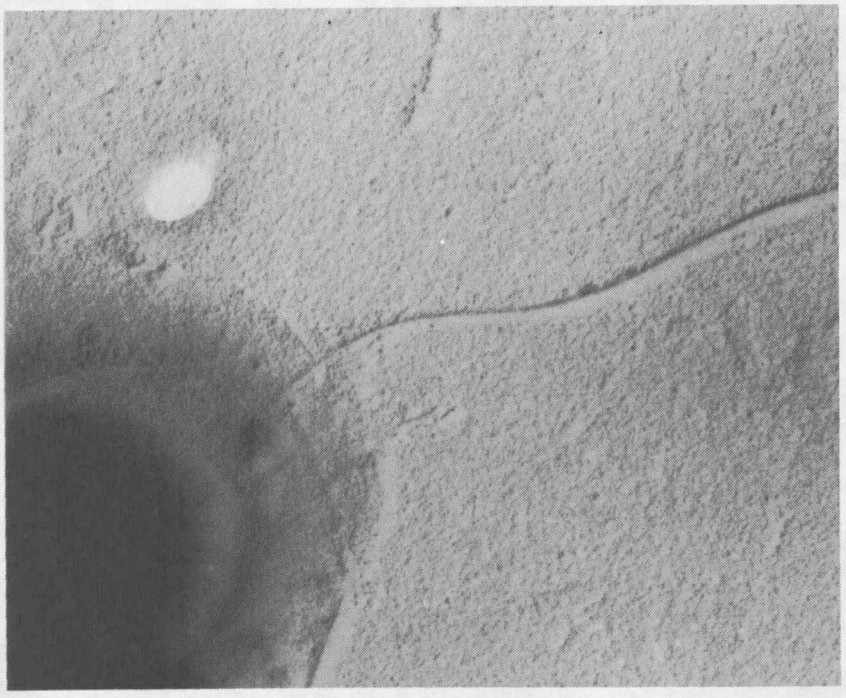


Plate 3. Bacillus Flagellum.
Electron micrograph 186,000X

nitrate and nitrite tests were made, both of which were negative. E. tions is about 15 minutes. To further characterize the Bacillus, coli gave a positive reaction as a control. From these tests, morphological studies, and through use of Bergey's Manual of Determinative Bacteriology (1957) and The Genus Bacillus (Gordon, 1973) it was determined that the organism of study is a strain of B. stearothermophilus. Table 1 presents comparative characteristics of Thermus and B. stearothermophilus.

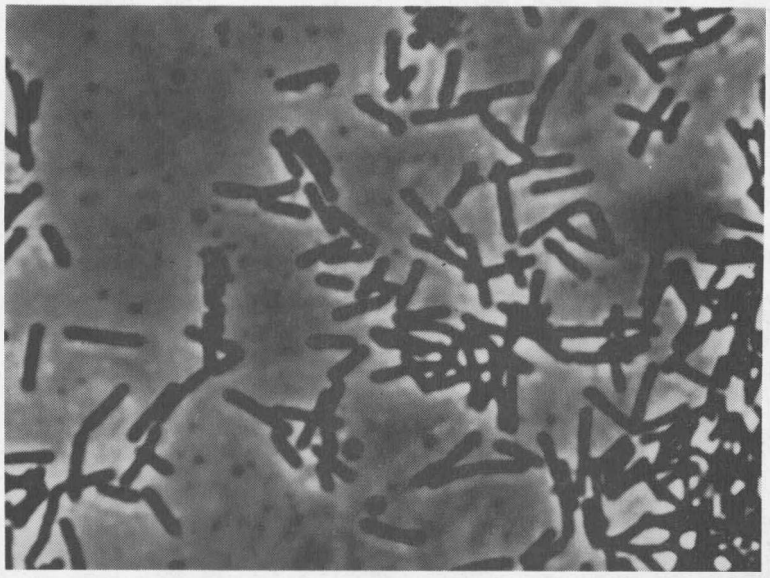
Culture of Bacillus. The basal salts plus yeast extract and tryptone medium (pH 7.5) were used routinely for B. stearothermophilus. Nutrient agar gave comparable results in one experiment and would be a simpler preparation. It was found that the most reproducible results were obtained when the plates were dried with lids ajar at 70 C for about 1 1/2 hours or left without sealing on the laboratory bench at room temperature for several days before use.

Characteristics of Thermus.

Morphology. Thermus was originally described as filamentous at its optimum temperature of 70 C. However, at lower temperatures filamentation decreases. Electron micrographs (plate 4) confirm this description. Electron microscopy also showed a lack of flagella and spores and confirmed tangling and filamentation at high temperatures.

Cultures of Thermus. Points of consideration in the development of solid medium for growth of Thermus at 70 C included:

a) Requirement for a firm, non-melting medium for incubation at 70 C.



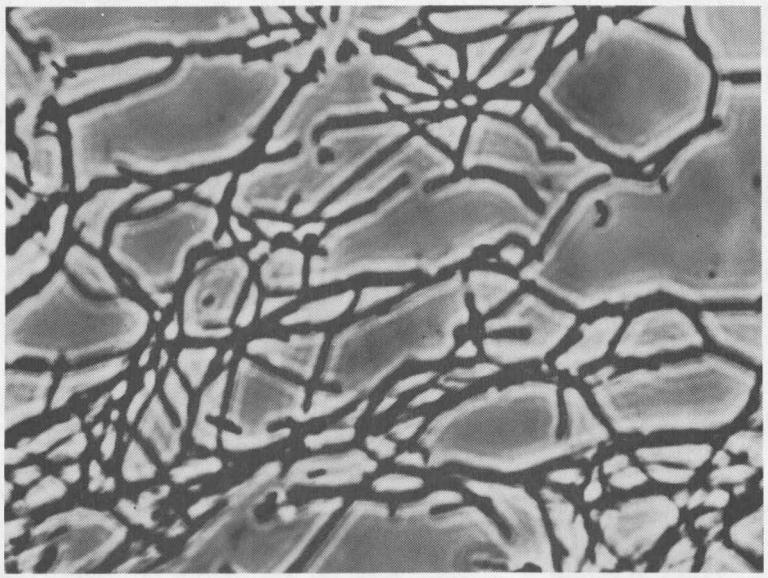


Plate 4. Thermus 55 C 100X (above)
70 C 50X (below)

Table 1. Summary of characteristics of B. stearothermophilus and Thermus T2.

	Bacillus	Thermus
Gram-stain	V	-
Spores	+	-
Flagella	+	-
Presence of functional lac operon	-	+
Filamentation at 58 C	-	-
Filamentation at 70 C	-	+
Optimum Growth Temperature	55 C	70 C
Optimum Growth pH	7.5	7.8
Generation Time	15 min	1 hour (70 C) 2.5 hours (55 C)

V = Variable
+ = Presence
- = Absence

b) Prevention of drying at the high temperature is indispensable, yet media that is too moist allows colonies to run together, preventing accurate counting.

c) Clear medium to allow use of the automatic colony counter was desirable.

d) Gelrite and the high concentrations of $CaCl_2$ and $MgSO_4$ required for its polymerization precipitates high salt from any medium in which both are used, especially at pH above 7;

e) Salts and pH 7.8 are required for growth of Thermus.

f) Media containing Gelrite solidifies at a higher temperature than agar and must be poured above 60 C. Once solidified, it cannot be melted again.

The medium of choice for growth of Thermus on solid support at 70 C became Johnson's with Gelrite as the salt content was nutritionally sufficient and yet low enough to allow the use of additional high concentration of $CaCl_2$ and $MgSO_4$ required for use of Gelrite. In spite of the disadvantage that the medium had to be poured at 65 C, Gelrite provided the clearest product and thus allowed use of the automatic colony counter. Better growth (colony number) was observed with Gelrite as a gelling agent by Lin and Casida (1984). The results of this study did not corroborate the Lin and Casida results as to an increase in colony number increase.

Growth of Thermus in broth at 70 C could be attained in either Johnson's or Ulrich's but only in shaken culture. The ten-fold dilutions required for plate counts were only successful when diluent was at pH 7.8.

A typical growth curve determined spectrophotometrically indicated a generation time of approximately 150 minutes at 55 C or 60 minutes at 70 C similar to observations of Ulrich (1971). However, turbidity or absorbance and plate counts showed a discrepancy in logarithmic phase perhaps because of filamentation and consequent prevention of cell division or tangling. Reproducible results in experiments involving plate counts were obtained only with O.D. $\leq$ 0.500 and with precisely controlled conditions (pH, medium, plating).

With generation time of one hour and a long lag phase for Thermus an unreasonable time was required to achieve log phase at a cell density appropriate for experimental work. This difficulty was

overcome by inoculating broth from a single colony and allowing the newly inoculated culture to grow overnight at the appropriate temperature (55 C or 70 C) with shaking (day #1). If growth had occurred, a dilution was made in growth medium, the culture grown again at the appropriate temperature with optical density monitored, and the culture removed during log phase to the refrigerator (day #2). The culture could then be taken from the cold the day of the experiment (day #3), diluted and incubated again. With very little lag phase the culture quickly re-entered log phase.

B. Quantitative Results of Killing by Irradiation

The killing of a microorganism is defined for these experiments as the loss of its ability to initiate a colony. Within a population the individuals that have experienced enough chemical events to result in unrepaired changes are inactivated or "killed". It is assumed that the damaging events occur randomly and independently in the susceptible groups with the probability of such an event proportional to the dose of radiation per unit time. Target size is generally indicated by the slope of the killing curve on semi-log paper. As long as the dose rate is constant organisms with the same target (genome) size can be compared and the relationship between dose and killing analyzed.

The reference E. coli strain (AB1157) of Figure 3 is a UV resistant wild type strain. Data used for generation of the reference curve was obtained under identical conditions using the same facilities as the Thermus and Bacillus stearothermophilus irradiation

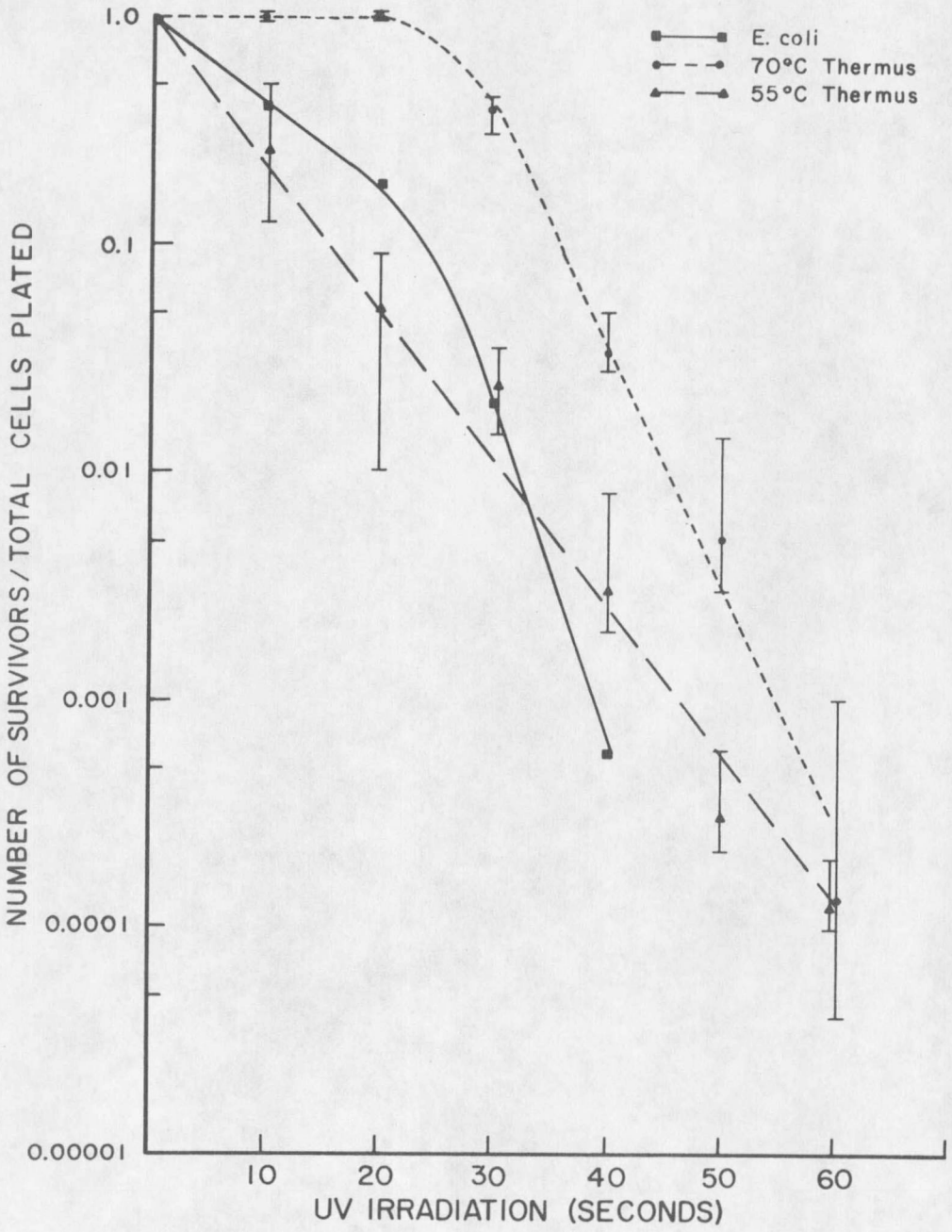


Figure 3. UV Survival: E. coli, Bacillus, Thermus 55 C and Thermus 70 C.

experiments in the laboratory of Guylyn Warren, MSU. Figure 3 also shows the survival curves generated for Thermus grown at 55 C and 70 C.

Bacillus grown at 55 C prior to irradiation shows a characteristic single-hit curve with exponential decay as does Thermus grown at 55 C. However, Thermus grown at 70 C prior to irradiation displays the characteristic "multiple-hit" curve with a shoulder with the exponential decay indicative of killing only after 30 seconds of exposure to UV. Furthermore, the experiments in which Thermus was grown at one temperature and switched to the other temperature prior to irradiation indicated that the switch from lower to higher temperature induced the mechanism responsible for the multiple-hit curve, that the induction was complete within two hours after the temperature switch (Figure 4), and that this mechanism remained active through the four hours monitored and shown here. (Data not presented demonstrated that the acquired characteristic was active in a late log to stationary phase overnight culture grown at 70 C. Additionally, the mechanism appeared active for at least three hours after switching from 70 C to 55 C.) Graphs present an average of three replicative experiments, each experiment including three plate counts per point. Error bars represent high and low values at each point.

C. Exposure to UV and MC.

One explanation of the multiple-hit survival curve of Thermus T2 could be induction of a DNA repair system. This was tested by exposing dilutions of Thermus on plates to UV and then incubating the

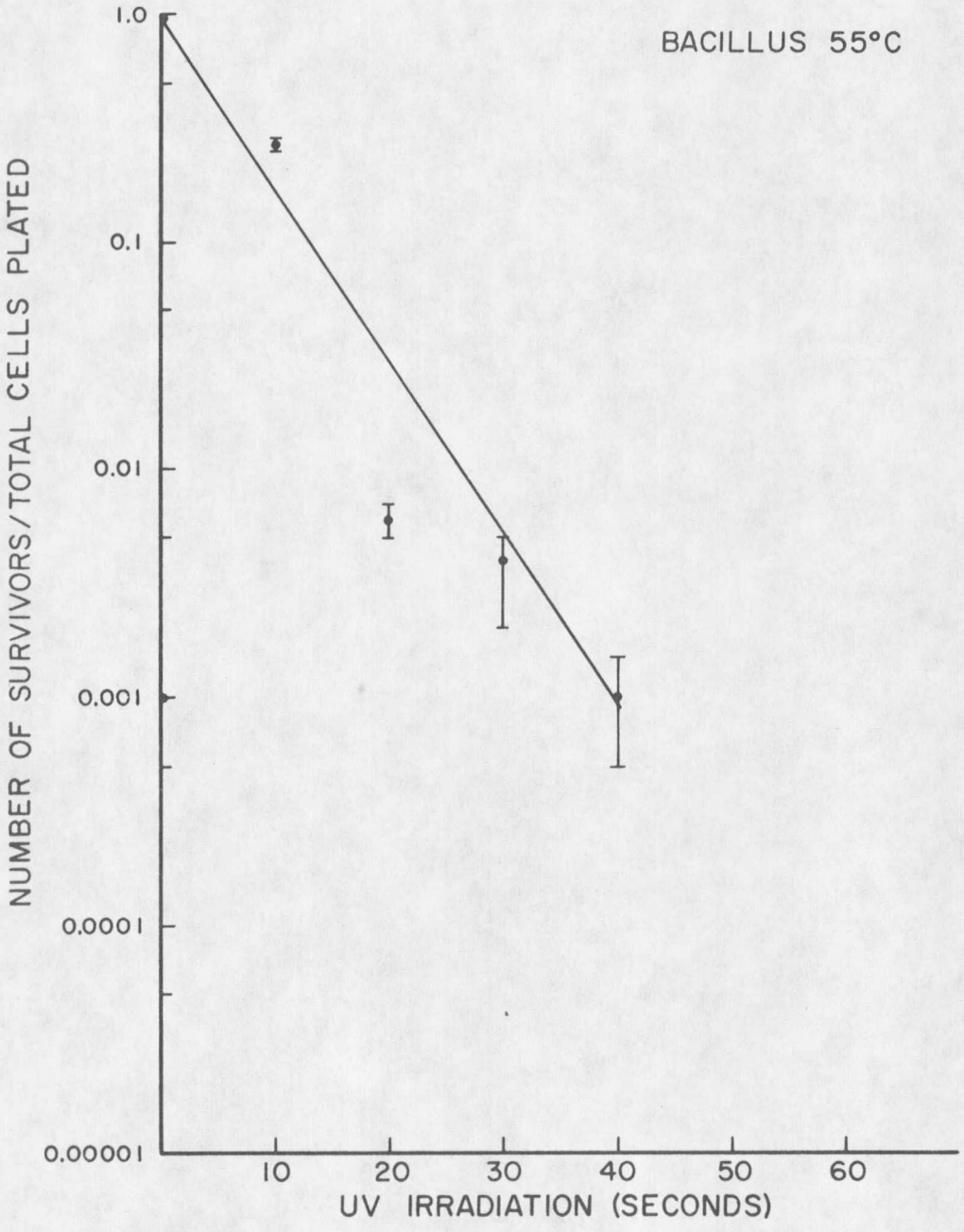


Figure 4. UV Survival: Thermus 1, 2, 3 hours after transfer from 55 C to 70 C.

plates with Mitomycin C in discs and observing, after colonies had grown up, the diameter of the kill zone surrounding the discs. If UV exposure induced a DNA repair system, increased resistance to Mitomycin C would be expected. Alternately, a log phase culture of Thermus was exposed to Mitomycin C for a short determined length of time, the mutagen washed out, cells plated, and then irradiated with increasing doses of UV. The resulting survival curve would indicate whether pretreatment with Mitomycin C increased resistance to UV.

In one experiment with MC discs there was not a conclusive zone size difference. In one experiment with triplicate samples of MC pretreatment followed by UV, the 55 C survival curve developed a shoulder similar to the 70 C cultures (Figure 5).

D. Quantitative Results of Photoreactivation and Direct Light Repair Following UV Treatment.

Table 3 presents the results of photoreactivation experiments on a wild type E. coli and Bacillus stearothermophilus under the conditions described in the Methods section of this thesis.

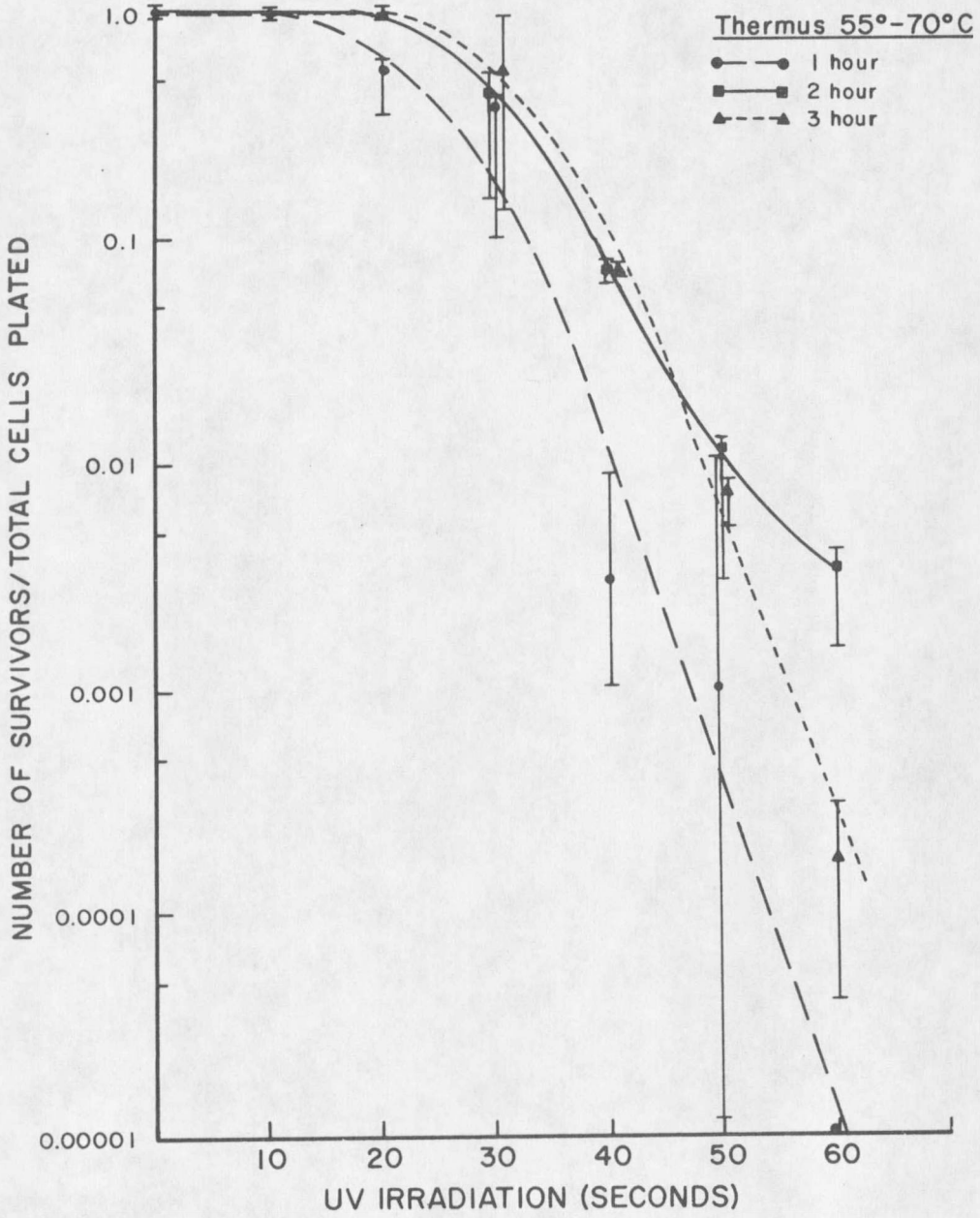


Figure 5. Mitomycin C + UV Survival: Thermus 55 C.

Table 2. Photoreactivation: Bacillus stearothermophilus and E. coli

Treatment #	# Survivors	# Survivors/# Total*	% Photoreactivation
	Photoreactivation: Bacillus		
1	1.1×10^7		
2	$1.2 \times 10^{7*}$	1.00	
3	9.7×10^6	0.88	
4	4.7×10^5	0.04	
5	5.3×10^5	0.05	
6	9.4×10^6	0.85	96% ($\pm$ 3%)
	Photoreactivation: E. coli		
1	1.4×10^8		
2	$9.8 \times 10^{7*}$	0.7	
3	1.0×10^8	0.71	
4	3.3×10^7	0.24	
5	3.3×10^7	0.24	
6	9.5×10^7	0.679	97% ($\pm$ 2%)

E. coli, after 30 seconds of irradiation and subsequent survival of 2% of the population with no photoreactivation treatment show 97% reactivation after 30 minutes of exposure to a white incandescent photographer's bulb placed six inches from the culture. Bacillus with 20 seconds of irradiation and 5% survival of the nonphotoreactivated population demonstrates a 96% reactivation in response to exposure to a photographer's blue light bulb. The data presented in each case is the average of two experiments with samples taken in triplicate.

Photoreactivation of UV-dosed Thermus was also attempted (Table

3). Disappointingly, despite some variation of UV dosage and temperature during photoreactivation as well as use of light conditions that were successful in photoreactivating E. coli and B. stearothermophilus, photoreactivation of Thermus could not be demonstrated.

Table 3. Conditions of Attempted Direct light Repair

	E. coli	Bacillus	Thermus
Irradiation (seconds)	30	20	20, 30
Temperature	Ice	40 C	Ice, 37 C, 50 C
Light (time in minutes)	White (30,60)	Blue (15,30)	Blue (15,30)
Successful Repair	+	+	-

DISCUSSION

Thermally injured cells of any organism may die or repair the damage depending on the extent of damage and environmental conditions. DNA-damage can be repaired, the mechanism called upon depending upon the type and extent of damage and the environmental conditions as well as the capabilities of the organism. The relative roles of structural changes and inducible or non-inducible biochemical changes are being elucidated in the highly studied mesophiles. I examined thermophilic Bacillus and Thermus using variations on technical procedures now successful with their mesophilic counterparts. Once the technical aspect was affirmatively established for the thermophiles, an attempt was made to compare the data obtained with that published for thermophiles and comparative mesophiles.

The challenge of obtaining reproducible results with the filamentous Thermus was met primarily through the development of an appropriate medium for plate counts and the precise control of conditions including: a) time required for experimental manipulations, b) optimum pH and temperature of dilution and plating medium, and c) of critical importance, cell density. Use of cultures with $O.D_{550}$ above 0.5 produced highly erratic results. The degree of filamentation and tangling seen in these cultures could interfere with any plate count assay which required development of clones from single

cells.

Comparisons of thermophilic and mesophilic membrane proteins, ribosomes, and nucleic acids have shown the biochemical mechanisms of organisms growing in the two temperature ranges to be similar and thermostability to be inherent in molecular structural differences in the biochemical components of the cells. The restriction enzymes of each are active on the DNA of the other. DNA polymerase isolated from Thermus and compared to the E. coli polymerase indicates the subunits to be similar except for thermostability (Chien et al., 1976). Thermus DNA cloned into E. coli produces thermostable proteins (Tanaka, 1981; Nagahari et al., 1980). It is likely that the spectrum of DNA repair mechanisms present in thermophiles will also be similar to that observed among mesophiles. We could expect that photoreactivation could be quantitated in the thermophiles by recovery from UV after visible light treatment. Certainly if an inducible SOS-type repair system were present in a thermophile, we could expect a "multi-hit" UV survival curve. A positive indication of SOS-type inducible repair using agents known to induce the heat shock response in mesophiles at the higher of two temperatures within the thermophilic range would also demonstrate the possibility for a heat shock response similar to E. coli, including heat-inducible DNA repair, in the thermophile. Demonstration of the presence of heat shock proteins in thermophiles would also demonstrate the capability.

Stuy (1956) has reported that of 15 Bacillus strains investigated, only two showed good photoreactivation, four showed moderate

photoreactivation and the others were not photoreactivable. However, the strain of Bacillus appearing in Dr. Julian's MSU laboratory and used in these experiments was shown to be photoreactivable. We also showed that the strain can survive at 70 C. Direct light repair could not be demonstrated under these conditions with Thermus while the experiments were successful with E. coli and Bacillus stearothermophilus.

There is the question of whether UV-induced photoproducts are stable at 70 C. Apparently, Bacillus incurs 55 C-stable lesions which can be repaired by photoreactivation, but I have as yet, no information concerning dimer stability at 70 C. A positive result with photoreactivation in Thermus would have confirmed the presence of dimers as well as demonstrated repair by their disappearance. While some researchers consider UV damage to DNA to be included in one category (Hanawalt et al., 1979), others study more extensive forms of structural damage (Brash and Haseltine, 1982; Haseltine, 1983). Whatever the effect in Thermus, UV does "kill" the cell in doses similar to that of other bacteria. It seems valuable to continue the search for appropriate conditions for direct light repair at 55 C as well as 70 C.

Comparison of Thermus and E. coli survival after exposure to UV or the cross-linking agent, Mitomycin C, or Mitomycin C coupled with irradiation showed that dosages required for "kill" were similar for both organisms. When a survival or killing curve is a straight line passing through the origin, it is called a "single-hit" curve with a

single event responsible for the destruction of the viability of the organism. "Multiple-hit" curves have a shoulder near the origin before becoming linear because several events must accumulate in a viable cell (unit) before it is inactivated. (To determine the number of events required for inactivation, the straight part of the survival curve is extrapolated back to meet the ordinate axis.) The slope of the straight part of a multiple-hit curve has the same meaning as for a single-hit curve (Dulbecco, 1980).

Fifty-five C Bacillus and 55 C Thermus exhibited a typical "single-hit" survival curve while 70 C Thermus displayed the "multiple-hit" type in these experiments. The 70 C Thermus UV survival curve was found to be similar in shape to that of wild type E. coli (AB1157) and typical of inducible repair. The "multiple-hit" curve shows a shoulder which declines with UV kill after 30 seconds of treatment. However, when Thermus is incubated at 55 C a "single-hit" survival curve is generated, suggesting that the indicated inducible repair is not induced by UV at the lower temperature or is induced by temperature only. To further examine this problem, Thermus was grown at each temperature, switched to the other condition, and irradiated at various times. The results of these experiments indicate that the mechanism responsible for the "multiple-hit" curve is fully induced by two hours after a switch up to higher temperature and that the mechanism remains active at that temperature (confirmed by irradiating an overnight culture). It was also shown (data not presented) that the mechanism remains active for at least three hours following the

switch from 70 C to 55 C. Since the Thermus UV survival curve is related to the temperature at which the organism is growing, we have questions concerning inducibility of repair by other SOS-inducing agents at 55 C and 70 C and the relationship, if any, of the filamentation also seen at 70 C with this organism.

Mitomycin C was used as a pretreatment followed by the standard UV treatment of both 55 C and 70 C cultures to provide confirmation of induction of DNA repair. Here the 70 C Thermus "multiple-hit" survival curve remained unchanged while the 55C Thermus survival curve indicates inducibility to be present. Apparently, the inducing signal cannot be generated by UV damage under our conditions at 55 C while a positive result is obtained with the cross-linking mutagen.

Nucleoid structure of Thermus has not yet been examined. However, Lossius et al. (1983) have reported differences in the sedimentation coefficients of the envelope-free nucleoids of several strains of E. coli carrying mutations in the uvrA, uvrB, and rec A genes when induced by Mitomycin C and propose that these structures are repair intermediates. The examination and identification of nucleoid structures in Thermus mutants will be an exciting area of research, an integral part of the illucidation of the relationship of heat tolerance and inducible DNA repair.

The complex set of operons coordinately induced in E. coli by a shift to higher temperature demonstrates the principle by which Thermus may induce DNA repair and protective structural nucleoid proteins in response to heat. Thermus grows well over the temperature

range studied, presumably adjusting protein synthesis and degradation in response to temperature change. Although the temperature range is different for E. coli and Thermus, the changes for each will probably be shown to be a continuum over the entire range at which the organism survives, i.e., we can expect the proteins associated with the DNA to change with heat increases. The functional significance of heat shock proteins is unknown. Nucleic acid stability, DNA repair, and degradation of deleterious proteins have been postulated as functions. For Thermus it is reasonable to expect that maintenance of a stable DNA helix at high temperature requires the presence of associated proteins induced by heat as well as the other stabilizing factors suggested in the literature. DNA repair and degradation of proteins may also be possible. Explanations for the variation in UV survival curves for Thermus at two temperatures could be that for some reason the inducible repair system is not inducible by UV at the lower temperature or that filamentation itself contributes to the shape of the curve. Perhaps a better explanation is that the higher temperature itself induces a repair system capable of repairing UV lesions.

As discussed earlier, filamentation in E. coli is involved in mutations which confer sensitivity to many mutagens. How the intricate relationship of cell division and its molecular control in lon and sul mutants is related to repair and recombination remains cloudy. In that organism, there is more than one pathway to filamentation. Certainly temporary filamentation can be induced along with SOS traits (Witkin, 1976). In Thermus filamentation was found when

the organism was grown at 70 C; UV induction of the repair system we are observing is not required. If filamentation is a survival mechanism at high temperature, the cell may have more time for repair before another cell division. (Also, the larger surface area of the filamentous form may be able to utilize the lower amounts of oxygen in 70 C fluid more efficiently.)

Studies of constitutive and inducible repair, temperature-induced filamentation, discoveries of the single strand binding protein and the principle uvr proteins as well as regulatory mechanism of the rec A and lex A gene products of SOS repair systems, and the heat shock response have been productive in E. coli because of isolation and study of mutants demonstrating these coordinately induced activities. The isolation of repair-deficient mutants of Thermus is essential. The suicide method once employed for isolating auxotrophic mutants of E. coli (Davis, 1948) has been employed by Sancar and Rupert (1978) in the isolation of photoreactivation- and dark-repair-deficient mutants from E. coli and could produce repair-deficient mutants from Thermus for the continuation of this line of study. Too, auxotropic mutants could be isolated following mutagenization and the mutation rate observed as a rate of reversion to prototrophy. Dr. Emmet Johnson (personal communication) has observed a very high mutation rate within the lac operon of Thermus at 70 C (not examined at 55 C). Determination of the rate of mutability in Thermus at 55 C and 70 C would clarify the question of involvement of error-prone DNA repair in the 70 C Thermus result. Warner (1983) recently observed that 70 C

Thermus does demonstrate repair in uracil-DNA glycosylase and apurinic endonuclease activities, both of which are known to have roles in heat-damaged DNA repair in E. coli but that the levels detected are no greater than that found in E. coli.

Mutation in the lon gene is responsible for over production of capsular polysaccharide filamentation upon irradiation as well as defective for bacteriophage lysogenization and inheritance of F plasmids. Lon mutants are also deficient in degradation of abnormal polypeptides (Gottesman and Zipser, 1978) and it is thought that the multiple in vivo phenotypes are derived from their degradation-deficient property. It has been shown that mutations in lon decrease the rate of degradation of a mutant sigma subunit of RNA polymerase in vivo (Grossman et al, 1985) (and suggested that the lon$^-$ allele may affect the rate of the sigma synthesis as well). Trasler and Gottesman (1984) suggest that the defect in regulation of capsule formation found in lon cells may be the result of degradation of a positive regulator of capsule synthesis. It is now known that the lon gene product of Escherichia coli is a heat shock protein (Goff et al., 1984; Phillips et al., 1984).

The inducing signal of heat shock in other organisms is unknown. Why all the phenomena associated with SOS-repair, prophage induction, and the heat shock response can be produced by such different agents as UV irradiation, ethanol, or thymine starvation has not yet been explained. Inhibition of DNA synthesis (Radman, 1975) and production of low molecular weight products of DNA degradation (Pardee, 1975) are

suggested explanations. That known inducing factors causing relaxation of DNA molecules (superhelical in living organisms) through single strand breaks (x-rays), excisable damage resulting in single strand breaks (UV and Mitomycin C) or inhibition of DNA synthesis and prevention of ligation of single strand breaks could cause repressor proteins reacting with superhelical DNA to dissociate from the DNA molecule with a DNA configurational change (relaxation) permitting transcription of the repressed genes has also been suggested as an explanation (Luchnik, 1979). The binding of proteins to DNA is finely balanced (topoisomerase vs. local melting for RNA polymerase) to provide for a rapid response to change in environmnetal conditions. By this theory superhelicity would control binding of the E. coli lex A product to the operator site in the rec A operon and dissociation would permit transcription of the SOS genes; other functions could be induced in the same fashion. One explanation for our data is that heat causes the superhelical relaxation required for induction. The extent of DNA winding is temperature-dependent and a 15 C increase in temperature (heat shock) unwinds DNA one base pair per 200 base pairs (Travers and Mace, 1982) (which is equivalent to the maximum transcriptional level in E. coli with 1,500 polymerase molecules unwinding the 15,000 base pair genome). Travers and Mace investigated this idea in E. coli. Their studies showed that inhibition of the B subunit of DNA topoisomerase II induces proteins which are heat shock proteins but not the full set of known hsps. The heat shock phenomenon is suggested to be protection against relaxation. However, they con-