# DNA REPAIR IN *BACTEROIDES FRAGILIS* Bf-2

by

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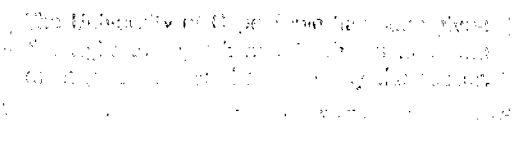

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"We like to think of exploring in science as a lonely, meditative business, and so it is in the first stages, but always, sooner or later, before the enterprise reaches completion, as we explore, we call to each other, communicate, publish, send letters to the editor, present papers, cry out on finding."

Lewis Thomas
in "The Lives of a Cell - Notes of a Biology Watcher"
Published by Allen Lane, Penguin Books, 1980.

## CONTENTS

## ABSTRACT

Repair deficient mutants of Bacteroides fragilis have been isolated in order to study the responses of this organism to various DNA damaging agents at the physiological and molecular levels.

Two types of mutants were isolated by ethyl methane sulphonate mutagenesis of B.fragilis followed by selection for sensitivity to mitomycin C. One mutant (UVS9) showed sensitivity to both mitomycin C and far-UV irradiation. The other (MTC25) was more sensitive to mitomycin C than UVS9, but showed wild-type resistance to UV radiation. Both mutant strains had wild-type resistance to methyl methane sulphonate.

Aerobic liquid holding recovery occurred in all three strains. Under anaerobic conditions the wild-type and MTC25 strains showed a decrease in survival during post-irradiation holding in buffer, while the UVS9 strain showed a low level of liquid holding recovery.

The MTC25 and UVS9 mutants had reduced host cell reactivation and UV-induced phage reactivation capability compared to the wild-type. Phage reactivation was $H_2O_2$-inducible in all three strains. The MTC25 mutant was induced to the same extent as the wild-type above their respective basal reactivation levels, and the UVS9 to a lesser extent.

Removal of pyrimidine dimers from irradiated DNA *in vivo* was examined using *Micrococcus luteus* pyrimidine dimer-specific endonuclease digestion of the extracted DNA followed by alkaline agarose gel electrophoresis. The wild-type strain showed complete removal of all endonuclease sensitive sites during 1-2 h post-irradiation holding in aerobic or anaerobic buffer. The MTC25 mutant removed approximately 60% of the sites under anaerobic conditions and 100% in air, while the UVS9 mutant showed reduced levels under both conditions.

Dimer removal was retarded but not inhibited by pre-irradiation treatment with chloramphenicol indicating a constitutive but partially inducible system.

Analysis of the strains for a dimer removal system involving an incision, excision and resynthesis mechanism was carried out using alkaline sucrose gradient sedimentation of irradiated DNA sampled during post-irradiation incubation of cells.

Under anaerobic conditions the wild-type and the two mutant strains showed levels of strand breakage and resynthesis comparable to their relative loss of pyrimidine dimer endonuclease-sensitive sites assayed under the same conditions. Under aerobic conditions, the UVS9 and MTC25 mutants generated far fewer strand breaks than the wild-type.

There was decreased survival of irradiated cells of all three strains to the same extent during post-irradiation plating on medium containing caffeine. Holding of irradiated cells in caffeine buffer before plating on caffeine-free medium also lowered the survival. Unirradiated cells treated under the same conditions were not affected. Caffeine completely inhibited dimer removal from irradiated wild-type cells as assayed by dimer-specific endonuclease digestion and alkaline agarose gel electrophoresis. It did not inhibit either strand breakage or resynthesis in UV-irradiated DNA during anaerobic holding. Caffeine exerted a direct strand-breakage effect equally on irradiated and unirradiated DNA. In unirradiated wild-type cells, the number of strand breaks in the DNA increased with increasing concentrations of caffeine.

Mitomycin C generated a DNA cross-linking response as well as a major degradative response in B.fragilis DNA treated with a lethal dose. Plasmid DNA was more resistant to the latter effect than chromosomal DNA.

# CHAPTER I

## GENERAL INTRODUCTION

The genus Bacteroides is comprised of a major group of obligately anaerobic, Gram-negative, non-spore-forming bacilli. Both pathogenic and non-pathogenic strains are commonly found in the alimentary canal of warm-blooded animals, and the pathogenic strains are the single most important group of anaerobic bacteria infecting humans. The morphological and physiological characteristics of this genus with special reference to its clinical identification and natural habitat have been comprehensively reviewed by Salyers (1984).

Members of the Bacteroides group usually occur in mixed infections, but the species Bacteroides fragilis is the predominant anaerobic micro-organism isolated from polymicrobial clinical infections (Gorbach and Bartlett, 1974; Duerden, 1980). The reasons for the enhanced virulence of this species over other Bacteroides species have been investigated (Reid et al., 1987; Rotstein et al., 1987; Verweij-van Vught et al., 1986) but have not yet been conclusively established.

Progress in molecular biology studies on this organism and on anaerobes in general has lagged behind that of aerobic organisms. There have been technical difficulties associated with working with anaerobes, and genetic

studies of Bacteroides have until recently been hampered by the lack of suitable genetic exchange and gene cloning systems, as well as difficulties in isolating suitable mutants.

Two general approaches to the study of B.fragilis genetics and gene regulation have developed. The first approach is the direct study of exsisting B.fragilis genetic transfer systems in order to explain drug resistance variations, pathogenesis, and gene regulation at the molecular level. In aerobic organisms, genetic studies have been undertaken via the isolation of suitable mutants carrying genetic markers. These have been used together with naturally occurring genetic transformation and transduction systems, phage or plasmid vectors and transposons to study cellular physiology and biochemistry at the molecular level.

Studies of this nature in Bacteroides have, until recently, met with limited success. The main reason for this is that these organisms appear to have limited or unusual natural genetic transfer mechanisms. Phage lysogeny and transduction have not been demonstrated conclusively despite extensive searches (Booth et al., 1979; Members of this laboratory, personal communications), although pseudolysogeny appears to be common in Bacteroides species (Keller and Traub, 1974; Burt and Woods, 1977). In addition, there appear to be

unusual aspects related to mutagenesis in Bacteroides which are not observed in aerobic organisms (Chapter II) with the result that few stable mutants of Bacteroides have been available for genetic investigations.

The present status of genetic studies in Bacteroides is reviewed in Section A.

An alternative approach to studying gene regulation is the investigation of the physiological responses of the organism to DNA damage and environmental stress with a view to understanding the underlying mechanisms of mutagenesis, recombination and error-free/error-prone repair (Sections B and C). This is the approach taken in this study.

The research has focused on the isolation of suitable mutants for studying the responses of B.fragilis to various DNA damaging agents, particularly far-UV radiation and mitomycin C, and the investigation of possible links between repair, recombination, and mutagenic mechanisms at the molecular level.

## A. GENETICS

Resistance and sensitivity of B.fragilis to antibiotics is an important area of therapeutic research, and has served as a starting point for genetic studies on this organism. Recent reviews on antibiotic resistance in Bacteroides have been published (Cuchural et al., 1981; Smith et al., 1985).

The changing susceptibility of Bacteroides to antibiotics such as tetracycline, clindamycin and chloramphenicol strongly suggested the presence of resistance transfer factors in Bacteroides (Tally et al., 1984). The discovery of transferable antimicrobial resistance to clindamycin-erythromycin in Bacteroides (Tally et al., 1979; Privitera et al., 1979; Welch et al., 1979) both served to explain some of the observed anomalies in antibiotic sensitivity of these bacteria, as well as initiating a new field of study in B.fragilis at the genetic level. The work done in this area will be reviewed here briefly since these systems have provided the main basis for genetic studies of B.fragilis.

Two major types of genetic transfer systems have so far been observed in Bacteroides, one involving plasmid transfer, and the other being an apparently non-plasmid transfer system.

Several autonomous plasmids carrying a determinant for clindamycin-erythromycin resistance, and with the ability to transfer from B.fragilis donor cells to Bacteroides recipients, have been described. These are pBF4 and PBFTM10 from B.fragilis (Tally et al., 1982; Guiney et al., 1984a,b,c) and pBI136 from B.ovatus (Smith and Macrina, 1984). Furthermore, Robillard et al. (1985) and Shoemaker et al. (1986) have shown that the clindamycin-erythromycin ($Cc^r$-$Em^r$) resistance gene(s) on

two of these plasmids (pBFTM10 and pBF4) are located on transposons (Tn4400 and Tn4351 respectively), which are capable of transposition in B.fragilis and Escherichia coli. The Tn4351 transposable element has been found to carry a second antibiotic resistance gene, that of resistance to tetracycline ($Tc^r$). The $Cc^r$-$Em^r$ genes are expressed only in Bacteroides and not in E.coli and $Tc^r$ is expressed only in aerobically grown E.coli and not in anaerobically grown E.coli or in Bacteroides spp. (Guiney et al., 1984b,c; Shoemaker et al., 1985).

The coincidental resistance to the macrolide, lincosamide, and streptogramin B-type antibiotics ($MLS^r$) carried by the three plasmids described above has been found to be localised on a single EcoR1 restriction fragment from each plasmid, and considerable homology has been found between their sequences (Guiney et al., 1984a; Shimell et al., 1982; Smith, 1985). Furthermore, the amino acid sequence, deduced from the DNA sequence, of the $MLS^r$ gene (ermF) from the pBF4 plasmid showed remarkable homology with those of erm gene products from 6 gram-positive bacteria (Rasmussen et al., 1986) indicating inter- as well as intragenic evolutionary transfer of genetic material.

A second type of genetic transfer has been found to occur in Bacteroides without any detectable plasmid involvement. Tetracycline resistance has been found to be transferred independently of resistance to clindamycin. The transfer properties and increased levels of resistance are

inducible by sub-inhibitory concentrations of tetracycline (Privitera *et al.*, 1979).

Evidence of non-plasmid associated genetic transfer was also detected by Macrina *et al.* (1981). *In vitro* filter matings were used during the transfer of tetracycline and clindamycin resistance from *B.fragilis* to a plasmidless strain of *Bacteroides uniformis* (Mays *et al.*, 1982; Smith *et al.*, 1982).

Recently, *Bacteroides* plasmids have been cloned in *E.coli* and shuttle vectors have been developed that allow transfer of DNA from *E.coli* back into *B.fragilis*, using broad host range plasmids to supply essential mobilization and conjugation functions (Guiney *et al.*, 1984b; Shoemaker *et al.*, 1986). Tally and Malamy (1986) have reviewed the current understanding of transferable resistance in *B.fragilis*.

An alternative approach to the establishment of genetic exchange systems in *Bacteroides* has been followed by Smith (1985), and he has reported a direct method for transforming *B.fragilis* through the use of polyethylene-glycol (PEG). The method should, in principle, allow for the use of standard gene cloning techniques and direct transformation of *Bacteroides* species. It has, however, only been successful with a particular *B.fragilis* strain and modifications are reported to be in progress.

Transformation studies in our laboratory with other Bacteroides strains using Smith's (1985) method have so far been unsuccessful.

Recently, a gene bank of B.fragilis (strain 01) has been established using the pEcoR1 cloning vector. Two B.fragilis genes have been cloned, expressed and characterized in E.coli. These are the glnA gene (Southern et al., 1986; Southern et al., in press), and the recA gene (Goodman et al., in press). The significance of the cloning of the recA gene is discussed in greater detail below (Section B; Section C.3.2).

## B. STUDIES ON DNA DAMAGE AND REPAIR SYSTEMS IN B.FRAGILIS

Major advances have been made in molecular biology studies of aerobes through investigations of their responses to environmental stress. In particular, studies on the effect of UV irradiation and UV-induced phage reactivation have assisted in the characterization of DNA repair, recombination and mutagenic systems in several gram negative aerobes, especially E.coli and Salmonella typhimurium (Bernstein, 1981; Walker, 1984). Comparative studies involving responses to heat, radiation, alkylating agents, oxygen and oxidizing agents have further served to elucidate the various regulatory networks induced by these stress factors and DNA damaging agents, as well as the inter-relationships between them. Adaptive responses to alkylation and oxidation damage have recently been

reviewed by Demple (1987), and the heat shock response by Lindquist (1986). Walker (1984; 1985) has comprehensively reviewed the present understanding of inducible DNA repair systems.

Similar studies on the effects of environmental stress factors on anaerobic organisms were, until recently, largely neglected and the responses of the extremely well-studied E.coli taken to be typical of bacteria in general.

However, recent work on B.fragilis has shown the presence of several novel and interesting DNA repair phenomena, and a comprehensive review on this topic has been published by Woods and Jones (1986). Aspects of the physiological responses of B.fragilis to UV radiation, oxygen and hydrogen peroxide which are relevant to this study will be highlighted here.

Inactivation of aerobic bacteria by far-UV radiation has been shown to be independent of the presence of oxygen (Zetterberg, 1964; Webb and Lorenz, 1970; Webb, 1977). However, fresh clinical isolates of the B.fragilis strain Bf-2 were found to be more sensitive to far-UV irradiation (254 nm) in the presence of oxygen (Jones et al., 1980; Jones and Woods, 1981; Slade et al., 1981). This effect did not persist indefinitely, and was gradually lost over a period of more than six years in vitro cultivation and

transient exposure to oxygen. During this period the strain has become relatively aerotolerant compared to fresh isolates, and will survive exposure to air but will not grow aerobically. The oxygen/UV response has, however, still been observed in fresh clinical isolates of B.fragilis strains (Dr H. Goodman, Personal communication).

Inactivation of aerobic bacteria with near-UV (310-400 nm) radiation has been found to be enhanced by the presence of oxygen (Webb, 1977). Furthermore, E.coli has an inducible system which repairs most of the radiation damage (Peters and Jagger,1981). Slade et al. (1982) found that near-UV-induced radiation repair occurred in B.fragilis under aerobic conditions. The kinetics of repair followed the characteristic "V"-shape that was found in an E.coli AB1157 recA mutant (Peters and Jagger, 1981), and the induction threshold in B.fragilis (1.5 kJ $m^{-2}$) was substantially lower than that for E.coli (70-100 kJ $m^{-2}$).

The use of reactivation studies for observing the survival, under various host conditions, of phage treated with DNA-damaging agents has made a great contribution towards the understanding of the genetic systems involved in aerobic bacterial DNA repair (Devoret et al., 1975; Witkin, 1976; Bernstein, 1981).

Three inducible phage reactivation systems have been identified in B.fragilis to date, these being UV-induced

reactivation under anaerobic conditions, as well as novel $O_2$- and $H_2O_2$- induced phage reactivation systems. Slade et al. (1983a,b) characterized these systems physiologically. All three could be inhibited by chloramphenicol, and they appeared to be error-free repair systems since they did not result in the production of mutants. This differs markedly from the UV-induced reactivation (Weigle reactivation) of phage lambda (Weigle, 1953; Bernstein, 1981; Walker, 1984) which is one of the expressions of the SOS response (see Section C.3 below) and is associated with an increased mutation rate. It is not clear at this stage whether the three B.fragilis phage systems are distinct or whether they share common pathways.

In aerobic organisms, investigations of the proteins induced by agents which damage DNA, or interfere with cellular macromolecular synthesis and DNA replication, have made major contributions towards elucidating DNA repair regulatory systems. This is particularly so in the case of the many and varied induced SOS responses which will be discussed further (Section C3).

Schumann et al. (1982, 1984) investigated the induction of proteins by far-UV radiation, oxygen, and hydrogen peroxide. Their results showed that each treatment induced a particular set of proteins, but that certain proteins of similar $M_r$ (90,000 and 70,000) were induced by all three types of stress. These three agents also induced phage reactivation systems, and there is the

possibility of common enzymes being involved. However, similarity in $M_r$ does not prove identity of proteins, and functional identity needs to be established by further experimentation (Chapter V).

The experimental system used in the above work failed to detect a UV-induced protein of the $M_r$ (40,000) corresponding to the recA gene product of E.coli. Recent work in this laboratory, however, has resulted in the cloning of a recA-like gene from B.fragilis (Goodman et al., in press). Antiserum prepared against purified E.coli recA protein cross-reacted with both the cloned B.fragilis recA gene product(s) as well as with the corresponding two protein bands (37,000 and 39,000 Da.) in B.fragilis total cell extracts. No induced increase in the levels of these proteins could be detected on autoradiographs following irradiation with far-UV and $^{34}S$-methionine pulse-labelling (Dr J.Parker, personal communication).

Goodman et al. (1985) investigated the induction of proteins by B.fragilis bf-2 in response to heat-shock in relation to its responses to UV irradiation, oxygen and $H_2O_2$. The set of seven proteins induced by exposure to heat-shock ($M_r$ 125,000, 80,000, 74,000, 65,000, 56,000, 52,000, and 20,000) did not include any of $M_r$ equivalent to those induced by $O_2$ or $H_2O_2$. Although $M_r$ differences do not necessisarily disprove functional identity, it seems likely that the heat-shock response in B.fragilis is

regulated by a different induction network from the other stress-related systems described previously. This proposal is supported by the fact that heat-shock was not able to induce phage reactivation as was the case with UV-, $O_2$-, and $H_2O_2$-induced damage.

The effects of UV radiation on macromolecular synthesis in B.fragilis have been investigated by Schumann et al. (1984). It was found that UV irradiation of cells under replicating conditions caused the immediate and extensive degradation of the existing DNA which continued for 40-60 min and during which time DNA replication was decreased but never completely inhibited. The degradation was inhibited by chloramphenicol suggesting that protein synthesis was involved. The relationship between these findings and the repair of UV irradiated B.fragilis DNA, as reported in this work, is discussed in Chapter IV.

## C. UV RADIATION DAMAGE

The physiological responses reviewed above show that B.fragilis has several novel features in its response to UV-induced damage. The further study of these systems at the molecular level forms the main emphasis of this thesis.

Much of our present understanding of the responses of cells to UV radiation and other forms of DNA damage is derived from detailed physiological and molecular studies

on E.coli. A review of this work must, therefore, form the main comparative basis for studying any other system. However, where possible, information on other prokaryotic and eukaryoyic systems will be included.

DNA damage caused by UV radiation (254 nm) has been extensively studied. Besides being biologically important, the lesions generated in DNA by UV radiation at wavelengths approaching the absorption maximum of the molecule (260 nm) are amenable to experimental investigation since they are chemically stable and readily reproducible, and sensitive assays exist for their detection.

The major photoproduct generated by UV radiation is the intrastrand cyclobutane-type pyrimidine dimer (Patrick and Rahn, 1976), with the relative percentages of nucleotide composition (C<>C, C<>T, T<>T) of the dimers being influenced by the radiation dose (Setlow, 1968) and the base composition and sequence of the DNA (Gordon and Haseltine, 1982).

Recognition of these pyrimidine dimers forms the basis of cellular repair systems, and several models have been presented to explain how this is achieved. Certain enzymes appear to be highly specific for pyrimidine dimers alone, even in single-stranded DNA, and cannot recognize other types of DNA damage e.g. E.coli DNA photolyase

(Husain and Sancar, 1987), and M.luteus and phage T4 pyrimidine dimer DNA glycosylases (ref Chapter 3.1 and 1.3.2. below). It is proposed that in these cases there is direct recognition by the enzyme of the altered base. However, other enzymes, or enzyme complexes, have a broad damage recognition spectrum involving pyrimidine dimers and bulky chemical adducts (e.g. E.coli ABC endonuclease; Section 2.1.2 below). It is suggested that in these systems conformational changes in the damaged DNA are recognized. Kemmick et al. (1987) have used two-dimensional NMR studies of thymine dimers induced in double-stranded oligonucleotides to demonstrate that small distortions in the B-DNA structure do in fact occur at the dimer site.

Friedberg (1985) has presented a comprehensive overview of the presently recognized cellular responses to UV-induced DNA damage by dividing them into three major categories, viz

1) the direct reversal,
2) the excision, and
3) the tolerance of DNA damage.

Repair mechanisms (2) and (3) are the so-called "dark repair" processes to distinguish them from (1) which is light-dependent. Aspects of these three groups of responses to UV damage will be reviewed here with reference to their possible relevance to B.fragilis.

# 1 DIRECT REVERSAL OF UV-INDUCED DNA DAMAGE

## 1.1 ENZYMATIC PHOTOREACTIVATION

Enzymatic photoreactivation of pyrimidine dimers is an extremely widespread biological phenomenon and has been found to occur in a large variety of bacterial, plant, and animal systems (Rupert, 1975). It is a highly accurate and energetically favoured repair process by means of which a single cellular enzyme binds to the pyrimidine dimer and, in the presence of light (wavelengths 300 to 600 nm), cleaves it to produce monomers without any breakage of the phosphodiester backbone. Myles et al. (1987) have shown that the photolyase enzyme of E.coli does not repair C<>C pyrimidine dimers efficiently, while it does repair T<>T or T<>C pyrimidine dimers. Hays et al. (1985) have presented preliminary evidence that the phr gene plays a role in excision repair as well, since strains with phr deletions show reduced dark repair capacity. However, they do acknowledge that the deletion might map into an adjacent gene which could code for the required excision repair activity.

No physiological evidence for photoreactivation has, however, been detected using conventional techniques in B.fragilis, the only anaerobic organism studied so far (Jones et al., 1980).

## 1.2 PURINE/PYRIMIDINE INSERTION

Damage to DNA in cells frequently takes the form of the removal of purines from the sugar-phosphate backbone, which leads to depurinated DNA (apDNA). These apDNA sites may be recognized by DNA apurinic/apyrimidinic (AP) endonucleases (see below). An alternative enzyme system has, however, also been proposed by means of which there is direct re-insertion of purine bases into depurinated DNA. Livneh *et al*. (1979) have demonstrated such an 'insertase' activity *in vitro* using *E.coli* cell extracts. The appropriate deoxynucleoside triphosphate and $Mg^{2+}$ ions were specific requirements, and the reaction was inhibited by EDTA and caffeine.

The evidence for such a 'base-insertion repair' mechanism remains limited, and in some cases controversial (Kataoka and Sekiguchi, 1982). Furthermore, an equivalent system involving the recognition of apyrimidinic sites as generated by the removal of UV-induced dimers has not yet been demonstrated. Friedberg (1985), however, suggests that such a mode of repair of DNA through a single-step reaction would be highly favoured, and may well yet be discovered. The possibility that it could exist in *B.fragilis* which shows such a high degree of error-free DNA repair, as well as several novel physiological responses to DNA damage must, therefore, be considered.

## 2 EXCISION REPAIR OF UV-INDUCED DNA DAMAGE

Excision repair mechanisms for the removal of pyrimidine dimers from UV-irradiated DNA have been recognized in a variety of prokaryotic and eukaryotic cell systems. Examples of prokaryotes are E.coli (see below), Micrococcus radiodurans (Moseley and Evans, 1983), Micrococcus luteus (Zherebtsov and Tomilin, 1982), Neisseria gonorrhoeae (Campbell and Yasbin, 1984a), and Bacillus subtilis (Munakata, 1977). Excision repair of pyrimidine dimers in the eukaryotic systems Saccharomyces cerevisiae, Drosophila melanogaster, rodent cells in culture, and human excision repair systems have recently been reviewed by Friedberg (1985). Specific similarities and differences between these repair systems and those occurring in B.fragilis will be cited in the text of this thesis where relevant. A general review of the key enzymatic events which occur is given here.

Excision of pyrimidine dimers from UV-irradiated DNA may be considered to fall broadly into three sequential, enzymatically regulated events (Hanawalt et al., 1979; Lindahl, 1982). These are-

1) incision of the DNA at or near the dimer site,
2) excision and re-polymerisation of the damaged area,
3) re-ligation of the free ends.

Current thinking does, however, recognize a certain amount of overlap between incision and excision events in some cases, as will be indicated below (Friedberg, 1985)

## 2.1 DNA INCISION

Two groups of enzymes have been identified in the literature by means of which the DNA at or near the sites of pyrimidine dimers may be incised. They vary markedly in their recognition capability for damage, as well as in the site(s) of the incision(s) generated.

### 2.1.1 Pyrimidine Dimer-Specific DNA Glycosylases

Pyrimidine dimer DNA glycosylase enzymes catalyse the selective removal of pyrimidine dimers. The glycosylase may act together with an associated UV endonuclease activity to achieve a single incision of DNA at the site of a pyrimidine dimer, or there may be the sequential activity of a separate apyrimidinic endonuclease which generates a single nick in the DNA backbone at any site where the glycosylase has removed the dimer. These enzyme systems are reviewed in greater detail in Chapter 3.1. No evidence for the existence of pyrimidine dimer DNA glycosylase enzyme systems has yet been found in mammalian cells (LaBelle and Linn, 1982).

### 2.1.2 Damage Specific DNA Incision Enzymes

These enzymes or enzyme complexes incise not only UV irradiated DNA but also DNAs affected by a number of

agents which produce damage with a chemical structure broadly referred to as 'bulky DNA adducts' (Yeung et al., 1983a,b).

The most extensively studied system of this type is the E.coli uvr enzyme complex. The system is comprised of the products of the uvrA, uvrB, and uvrC genes acting together to incise the damaged DNA. Seeberg (1978, 1981) was the first to demonstrate the requirement for all three gene products through in vitro complementation studies of various uvr mutant cell extracts. Cloning, expression and amplification of the uvr genes (Sancar et al., 1981a,b ; Yoakum and Grossman, 1981) have led to efficient purification methods being developed for the enzymes involved (Yeung et al., 1986). In this way, the specific properties of the three gene products as well as their combined activity and regulation have been elucidated.

The uvrA protein has been shown to bind to both irradiated and unirradiated single-stranded DNA with equal efficiency, but shows a greater affinity for irradiated as opposed to unirradiated double-stranded DNA (Seeberg and Steinum, 1982; Yeung et al., 1983a,b). The uvrB protein cannot on its own bind to DNA, but in the presence of MgATP, is able to assist the uvrA protein to form a stable complex on a DNA molecule containing pyrimidine dimers (Yeung et al., 1983 a,b; Seeberg and Steinum, 1983). The uvrC protein then binds to the uvrAB complex and activates its endonucleolytic activity (Seeberg and Steinum, 1983).

Two nicks are then generated in the DNA, one at 7 nucleotides 5' to the pyrimidine dimer, and the other at 3 to 4 nucleotides 3' to it (Yeung et al., 1983a,b; Sancar and Rupp, 1983).

The regulation of the uvrABC genes will be discussed below (Sections 2.2 and 3).

## 2.2 EXCISION OF PYRIMIDINE DIMERS AND RESYNTHESIS OF DNA

After incision of the damaged DNA by either of the above-mentioned enzyme systems, exonuclease-catalysed excision of either the damaged nucleotides or the apyrimidinic sites occurs.

In the case of the single incision of the DNA by the sequential action of a DNA glycosylase and AP endonuclease, the dimerised pyrimidines are still covalently linked to the DNA (Chapter 3: Fig 3.1) and would appear to always require post-incision degradation of the DNA, probably through the action of 5' -> 3' exonuclease. This system has been studied in the greatest detail in phage T4 and has been reviewed by Smith et al. (1970), Pawl et al. (1976), and Bonura et al. (1982).

Excision repair mediated by the damage-specific E.coli uvrABC enzyme complex has been found to occur by two distinct but simultaneous mechanisms viz short and long patch repair. As was described above, the uvrABC endonuclease in vitro generates two incisions in the damaged DNA, approximately 12 to 13 nucleotides apart.

*In vivo*, the vast majority of excision repair events do, in fact, result in excised regions 20 to 30 nucleotides long (Kuemmerle *et al.*, 1981; Cooper, 1982) indicating that very little further degradation of the DNA outside of the damaged area is required for repair. Furthermore, the regulation of this type of repair has been found to be constitutive (Cooper, 1982), and repair synthesis is probably catalysed by DNA polymerase I, the product of the *polI* gene (Wahl *et al.*, 1983).

A very small fraction of the post-incision gaps generated during excision repair are, however, at least 1500 nucleotides in length (Cooper, 1982) indicating that extensive DNA degradation is, under certain circumstances, required for repair. The molecular mechanism or the physiological significance of long patch repair is not known. The system is, however, thought to involve DNA polymerases II and III (Cooper and Hanawalt, 1972) in addition to possibly DNA polymerase I (Cooper, 1982). Long patch repair, in contrast to short patch, is inducible, and is under the regulation of the *recA* and *lexA* gene systems (Section 3 below).

It is clear, therefore, that DNA polymerase I, with its exonuclease as well as polymerase functions (Kornberg, 1981) plays a central role in repair synthesis. However, total absence of all detectable repair is only observed *in vivo* when cells are mutant in all three DNA polymerases (Masker *et al.*, 1973).

Other enzymes which have been implicated in the degradation of incised UV-irradiated DNA in E.coli are exonuclease V, coded for by the recB and recC genes (Cooper, 1977), and exonuclease VII, coded for by the xseA gene (Chase and Richardson, 1974a,b).

The product of the uvrD gene has also been implicated in the latter steps of excision repair. Mutants in uvrD are sensitive to UV irradiation, but are able to perform normal incision of the DNA (Kuemmerle and Masker, 1980). Pyrimidine dimers are, however, released much more slowly, there is an increase in patch size, and also a delay in the closure of the gap (Rothman, 1978; Kuemmerle et al., 1982). The uvrD gene has been cloned (Kumura et al., 1983) and the gene product found to be very similar (if not identical) to DNA helicase II. On the basis of this information, Rothman and Fried (1984) have proposed a regulatory function for the uvrD gene product through unwinding of the template and thus controlling its interaction with DNA polymerase I. As is the case with the uvrABC genes, uvrD is inducible through the recA, lexA regulatory system (Section 3).

Evidence for the excision of pyrimidine dimers from B.fragilis comes from the work done by Jones et al. (1981) who showed that thymine dimers could be detected in the acid-soluble DNA fraction of UV-irradiated cells (Chapter II).

## 2.3 DNA LIGATION

Following excision and resynthesis of the damaged area of the DNA, the end of the newly-synthesized portion of the strand must be re-joined to the original parent strand. This is efficiently achieved through the action of DNA ligase (Konrad et al., 1973; Lehman, 1974).

## 3 DNA DAMAGE TOLERANCE (POST-REPLICATION REPAIR)

Two mechanisms by means of which the presence of dimers may be tolerated during cellular growth have been proposed.

### 3.1 TRANSLESION DNA SYNTHESIS

The translesion DNA synthesis model (Bridges et al., 1976; Echols et al., 1983) suggests that replication continues uninterrupted across the damaged site with decreased fidelity of the DNA polymerase (possibly DNA polymerase III) allowing random base insertion (Fig. 1.1A). This would result in an error-prone system of repair with the persistence of dimers in the original strands. Recent work by Livneh (1986) has shown that under in vitro replication conditions E.coli DNA polymerase III holoenzyme can insert nucleotides opposite pyrimidine dimers in UV-irradiated single-stranded DNA of phages M13 and ØX174 to a significant extent.

FIGURE 1.1(A): MODEL FOR TRANSLESION DNA SYNTHESIS. (Friedberg, 1985).

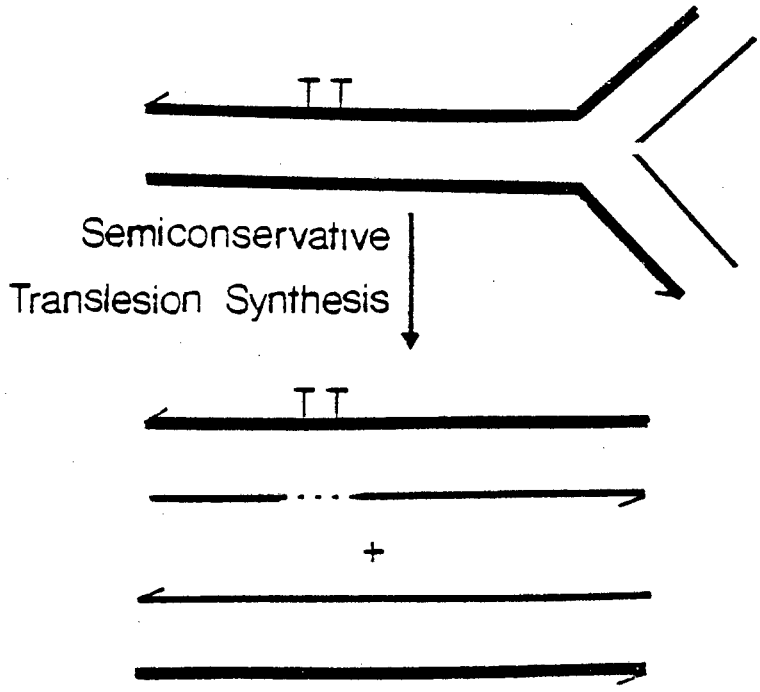
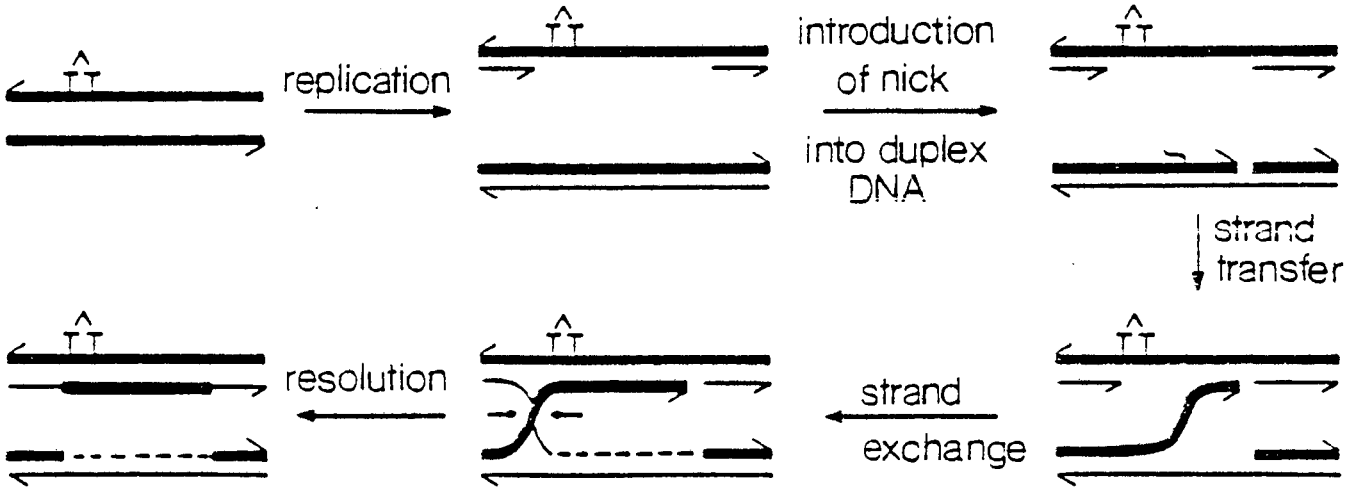



FIGURE 1.1(B): MODEL FOR DISCONTINUOUS DNA SYNTHESIS AND GAP FILLING. (Walker, 1985).



This model has not been as intensively studied as the model described in 3.2 (below) and information is still limited.

## 3.2 DISCONTINOUS DNA SYNTHESIS WITH GAP-FILLING

According to the current understanding of the two major dark repair processes in E.coli, the excision of pyrimidine dimers (as described above) is carried out chiefly in the unreplicated portions of the chromosome, is independent of growth medium, and is largely recA independent (Smith and Sharma, 1987).

Under replicating conditions, however, if the dimers (primary lesions) are not excised from the DNA, replication may proceed past them by stopping at the dimer sites and resuming replication just beyond them. Daughter-strand gaps (secondary lesions) are thus generated (Rupp and Howard-Flanders, 1968) which can be repaired by recombination with adjacent sister duplexes. This is usually referred to as postreplication repair, and is dependent on functional recA, recB, recF and lexA genes (Smith and Sharma, 1987). This mode of repair, therefore, involves the tolerance and gradual 'diluting out' of pyrimidine dimers through several rounds of replication (Fig. 1.1B).

### 3.2.1 The SOS regulatory system

The recA gene, as well as producing the protein required for DNA recombination, is also responsible for the induction of certain DNA repair genes. RecA is regulated by the lexA gene in E.coli and together they are responsible for the induction of a complex set of responses to conditions which damage DNA or inhibit DNA replication. Together these responses are termed the SOS phenomenon.

The SOS response has been reviewed by Witkin (1976), Gottesman (1981), Little and Mount (1982), Lindahl (1982) and Walker (1984, 1985). The relevant information reported in these reviews, as well as in some recent research papers, is summarized here.

Several diverse physiological responses of E.coli have now been recognized as being part of the SOS regulatory network. Amongst these are prophage induction, Weigle reactivation and mutagenesis of bacteriophage, UV sensitivity and UV mutagenesis (bacterial), cell filamentation, and DNA degradation following UV-irradiation.

The currently-held model for the mechanism by means of which the recA-lexA regulon is regulated is shown diagramatically in Fig. 1.2).

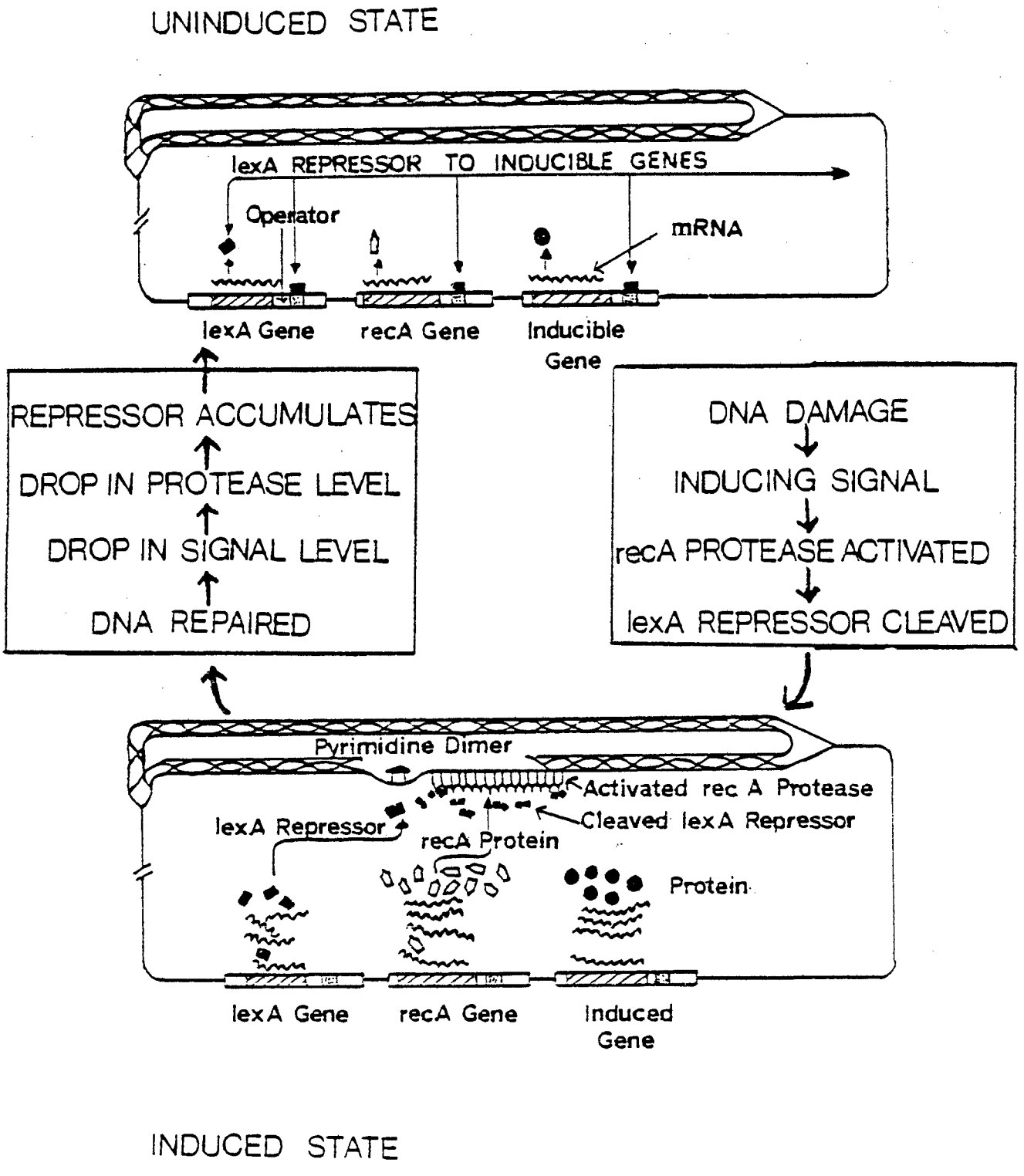



FIGURE 1.2: MODEL FOR SOS REGULATION

(Friedberg, 1985).

In the uninduced state, the product of the lexA gene is constitutively expressed in low amounts and acts as a repressor for a considerable number of unlinked genes, including the recA gene and the lexA gene itself. Others mentioned by Kreuger et al. (1983) are uvrA and uvrB (excision repair), umuC and umuD (induced mutagenesis), sulA (previously called sfiA; filamentous growth), and himA (site-specific recombination). Binding between the operator sequences (the so-called 'SOS' boxes) of the various genes and the lexA gene product has been shown (Brent, 1983). Although these genes are repressed, they are still able to produce a certain amount of the protein they encode constitutively, the amount of this varying from gene to gene. This accounts for the recA independent functions of the uvrA,B,C dimer excision system, as well as the availability of the recA protein for homologous recombination in uninduced cells.

When DNA damage or inhibition of replication occurs, the existing recA protein is activated to form a protease capable of cleaving the lexA protein as well as certain other proteins, such as the lambda repressor. The mechanism of activation is not clearly understood but is thought to involve binding of the recA protein, in the presence of ATP and single-strand binding protein (SSB), to the single-stranded gaps generated by discontinuous DNA synthesis at sites of damage (Cox and Lehman, 1982;

Howard-Flanders and West, 1983). The direct proteolytic action of the recA protein has, however, been questioned through the discovery that the lexA protein can, in fact, autodigest and that the recA protein could be acting as a cofactor in this reaction (Little, 1984).

Once the lexA protein has been cleaved, the repression of the SOS genes is relieved and they are coordinately induced. In particular, large amounts of recA protein are synthesized to continue the cleavage of lexA protein. The kinetics of the induction of the various genes varies according to the extent to which the lexA protein is bound to their operator regions, and in this way, the so-called 'fine-tuning' of the SOS response is achieved.

Repression of the system once again occurs with removal of the proteolytic inducing signal and the re-accumulation of lexA repressor.

SOS-like responses to UV-irradiation have been observed in several bacterial systems. Evidence for functionally conserved enzymes being active in divergent species of bacteria comes from complementation of recA protein functions in E.coli by a cloned Pseudomonas aeruginosa recA gene (Kokjohn and Miller, 1985). Conversely, a cloned E.coli recA gene was able to restore partially DNA repair and recombination capabilities, as well as induce

an SOS-like response (SOB), in a recE mutant strain of B.subtilis (Love and Yasbin, 1986).

Evidence for an SOS-like system in B.fragilis is, at this stage, still limited. Several of the characteristic SOS responses to UV damage reported above have not been found in this organism. These are filamentation (Jones and Woods, 1981), recA protein induction (Schumann et al., 1982, 1984; Dr J. Parker, personal communication), UV mutagenesis of bacterial cells (Jones, 1979), and Weigle mutagenesis (Slade, 1983). The topic of mutagenesis in B.fragilis will be dealt with in greater detail in Chapter 2.1.

Recently a recA-like gene from B.fragilis (Goodman et al., in press) has been cloned which is able to perform several of the functions of the E.coli recA gene product in an E.coli recA mutant (resistance to UV and methylmethane sulphonate, homologous recombination, and induction of lambda lysogeny). The protein coded for by this gene was found to be antigenically homologous to E.coli recA protein, constitutively expressed in B.fragilis, and showed no increase in level of expression following UV-irradiation as examined by pulse labelling of the proteins and autoradiography.

UV-induced (Weigle) reactivation of *B.fragilis* phage b-1 is the only induced SOS-like response which has been observed to date (Slade *et al.*, 1984a,b).

These findings in *B.fragilis* indicate that elements of an SOS-like system may be present and that further examination of repair responses at the molecular level could assist in clarifying aspects of this important regulatory network in an anaerobic organism.

# CHAPTER II

# ISOLATION AND CHARACTERIZATION OF BACTEROIDES FRAGILIS DNA REPAIR MUTANTS.

## SUMMARY

Mutants of B.fragilis sensitive to mitomycin C were isolated after mutagenesis with ethyl methane sulphonate. One mutant (MTC25) was markedly sensitive to mitomycin C but was unaffected as regards UV sensitivity; another mutant (UVS9) was sensitive to UV radiation but was only moderately sensitive to mitomycin C. Neither of the mutants showed increased sensitivity to methyl methane sulphonate. Caffeine decreased the survival after UV-irradiation of the wild-type, MTC25, and UVS9 strains by the same relative amount. Aerobic liquid holding recovery occurred in all three strains. Under anaerobic conditions, the wild-type and MTC25 strains showed a decrease in survival during post-irradiation holding in buffer, while the UVS9 strain showed a low level of liquid holding recovery. The MTC25 and UVS9 mutants had reduced host cell reactivation capability. The wild-type, MTC25 and UVS9 strains all showed UV- and $H_2O_2$-induced phage reactivation. The physiological characterization of the MTC25 and UVS9 mutants indicates that it is possible to differentiate between mechanisms for the repair of mitomycin C- and UV-induced DNA damage in B.fragilis.

## 2.1 INTRODUCTION

The isolation of mutants is of central importance to any investigation of bacterial genetic systems. In this study, repair deficient mutants were required in order to examine the responses of B.fragilis to DNA damage at the physiological and molecular levels, with a view to understanding the underlying mechanisms of stress-induced responses in an anaerobic organism. Isolation and characterization of these mutants was also considered potentially useful for future gene transfer and cloning studies.

### 2.1.1 MUTAGENESIS OF B.FRAGILIS

Some anaerobes have been reported to be particularly difficult to mutate by certain conventional mutagenic agents, and in particular by UV light. In earlier studies, lack of mutability was observed in Clostridium species (Sebald and Costilow, 1975), and in B.fragilis (Jones, 1979; Van Tassel and Wilkins, 1978). It is not clear at this stage just how widespread this phenomenon is since the range of anaerobic bacteria tested so far remains limited.

The reasons for the lack of mutability under certain circumstances are not precisely known, but are thought to involve the regulation of genes controlling DNA repair.

The cellular components required for mutagenesis in E.coli have been reviewed by Elledge et al., (1983) and Walker (1984). They point out that mutagenesis is not a passive process but requires the participation of several cellular components coded for by four different chromosomal loci- recA, lexA,umuC and umuD. Mutations in any one of these genes can make E.coli non-mutable by UV and a number of chemical agents.

Mutagenic agents have been divided into two classes by Miller (1983). Direct mutagens cause mutations by mispairing mechanisms involving either template or nucleotide precursors (e.g. chemical mutagens). Indirect mutagens (e.g. UV radiation) induce an error-prone postreplication repair system. However, as defined by UV immutibility, several organisms have been shown to lack error-prone repair. Among these are Neisseria gonorrhoeae (Campbell and Yasbin, 1984b), Micrococcus radiodurans (Sweet and Mosely, 1974), Streptococcus pneumoniae (Gasc et al., 1980), Streptococcus mutans (Sicard, 1983), and Haemophilus influenzae (Kimball et al., 1977; Setlow and Notani, 1981).

Campbell and Yasbin (1984b) have noted that these bacteria are also naturally competent, and suggest that error-prone repair together with efficient DNA uptake and transformation are not favoured genetically. They report

exceptions to this hypothesis as being the partially competent bacterium Bacillus subtilis which is also UV mutatable, and Salmonella typhimurium and Proteus mirabilis which are non-competent but lack aspects of error-prone repair (Walker, 1978; Hofemeister et al., 1979). B.fragilis is also non competent while appearing to lack a typical E.coli-like error-prone repair system (Chapter I).

Droffner and Yamamoto (1983) examined the effect of stringent anaerobic conditions on the mutagenesis of the facultative anaerobe, S.typhimurium. They found that an anaerobic environment caused the lack of expression of the bacterial recBC function and the recA regulatory functions for the SOS repair system which cause error-prone repair in this organism under aerobic conditions.

Van Tassel and Wilkins (1978) were, however, successful in isolating auxotrophic mutants of B.fragilis using mutagenesis with ethyl methane sulfonate (EMS), and Goodman et al. (1985) isolated heat-resistant mutants of B.fragilis using this mutagenic agent. Numerous mutants of Clostridium spp have been isolated over the past few years through EMS mutagenesis (Jones et al., 1985; Long, 1984), and Bowring and Morris (1985) have identified EMS and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as the best mutagenic agents for Clostridium acetobutilicum.

EMS and MNNG have been noted to be chemical mutagens which can cause mutations in E.coli in the absence of an active umuDC gene system and cellular SOS processing (Kato et al., 1982; Kato and Nikano, 1981; Schendel and Defais, 1980). The reason for this is thought to be that they cause mutagenesis through directly mispairing lesions. EMS thus appears to be one of the mutagens of choice for anaerobic conditions.

### 2.1.2 SELECTION METHODS

The selection methods applied to screening for UV repair deficient mutants are of two main types viz direct or indirect screening. Direct selection by exposure to UV irradiation itself has been used with success by numerous workers in the isolation of UV-sensitive mutants (e.g. Moseley and Copland, 1978; Yagi and Clewell, 1980). Okubo and Romig (1966) used a method of direct selection for non-host-reactivating mutants by screening for cell survivors following the exposure of mutagenised cells to UV irradiated phage.

Indirect selection is based on the fact that a correlation exists between between UV sensitivity and sensitivity to mitomycin C and various alkylating agents (Kersten, 1971). This has been reported in E.coli (Boyce and Howard-Flanders, 1964), M.radiodurans (Moseley, 1967), B.subtilis (Okubo and Romig, 1966), and S.typhimurium (Ames et al., 1973). There is evidence that this

correlation is due to the joint recognition of the lesions formed by a single repair mechanism. Details of the UV and the mitomycin C repair system are presented in Chapter I and the introductions to Chapters III and IV respectively.

Some organisms, however, do exist which are unable to repair UV damage, but can repair lesions induced by mitomycin C and other alkylating agents (Kohn et al., 1965; Setlow and Carrier, 1966). Two types of mutants of M.radiodurans have been isolated one of which has wild-type levels of resistance to UV damage but is mitomycin C-sensitive, and the other showing UV-sensitivity and only partial sensitivity to mitomycin C (Moseley and Copland, 1978). Two separate DNA excision repair pathways have been identified in this organism, one requiring a functional mtcA gene and the other functional uvsC, uvsD, and uvsE genes. Mutational blocks in both pathways must exist for an excisionless phenotype to be expressed (Evans and Moseley, 1983; Moseley and Evans, 1983)

Direct selection of UV-sensitive mutants using UV irradiation was not found to be successful in B.fragilis (Jones, 1979). Indirect selection with mitomycin C was therefore considered for this study. Mitomycin C has been reported to be chemically activated by reduction with hydrogen in the presence of a palladium catalyst, but it is highly unstable in this form and rapidly loses its DNA cross-linking ability (Szybalski and Iyer, 1967). Its

suitability as a selective agent under anaerobic conditions had to be established.

### 2.1.3 PHYSIOLOGICAL CHARACTERIZATION OF MUTANTS

Physiological characterization of mutants is an important preliminary study to the identification of the genetic loci involved. In B.fragilis, the establishment of routine inter- and intra-species genetic transfer systems of chromosomal genes is still at an early stage (Guiney et al., 1984a,b,c; Smith, 1985) (Chapter IA). Physiological characterization of mutants is, therefore, of great importance both in providing suitable recipients for genetic transfer experiments as well as in themselves providing information as to the genetic lesion involved.

As was mentioned previously, UV-sensitive mutants often show joint sensitivity to other damaging agents. RecA mutants of E.coli which are UV- and methyl methane sulphonate (MMS)-sensitive showed increased resistance to both damaging agents after transformation by cloned recA genes from a variety of bacterial sources such as Vibrio cholerae (Goldberg and Mekalanos,1986), P.aeruginosa (Kokjohn et al., 1985), and Rhizobium meliloti (Better and Helinski, 1983). Goodman et al. (in press) have shown that a cloned recA-like gene from B.fragilis is also able to increase the resistance of an E.coli recA mutant (HB101) to MMS. The joint recognition of UV-induced lesions and mitomycin C-induced damage and the role played

by excision repair genes in these processes was outlined in the previous section and in Chapter IC.

Excision repair genes have also been implicated in E.coli in the phenomenon of liquid holding recovery where irradiated cells held in buffer for a period of time before plating show increased survival (Harm, 1966). Ganesan and Smith (1968, 1969) showed that liquid holding recovery is observed in cells having functional uvr and polA gene products, but only if they have some other decreased repair capacity as is found in E.coli B or E.coli K12 recA mutants.

The ability of cells to reactivate UV-irradiated bacteriophage is another important physiological criterion dependent on the genetic status of the host cell. Devoret et al. (1975) showed that in E.coli there is a link between excision repair of UV-irradiated phage and the uvr genotype of the host cell, with reactivation of the phage depending on at least four host genes, uvr A,B,C,D. Monk et al. (1971) further showed that a functional polA1 gene was also required for host cell reactivation of phage to take place. Phage reactivation can also be dependent on recombination repair systems of the host cell (Devoret et al., 1975). The induction of increased levels of reactivation by UV irradiation of the host cells (Weigle, 1957) and by treatment with DNA damaging agents have furthermore been shown in E.coli to be dependent on the

*recA*, *lexA* inducible SOS error-prone repair system (Radman, 1975; Witkin, 1976). The significance of the phage reactivation characteristics of *B.fragilis* as reported by Slade *et al*. (1983a,b), have been discussed in Chapter IB.

## 2.2. METHODS

### 2.2.1 BACTERIA AND PHAGE STRAINS

These studies were done on a B.fragilis strain (Bf-2) and the phage b-1 which have been used in previous studies on the effects of far-UV irradiation (Jones et al., 1980; Jones and Woods, 1981; Slade et al., 1981, 1983a,b; Schumann et al., 1982, 1984). The B.fragilis strain was a clinical isolate identified by Dr L.V. Holdeman, V.P.I. and S.U. Anaerobe laboratory, Backsburg, U.S.A. (Jones, 1979). Phage b-1 was originally isolated from sewage by Prof. D.T.Jones, U.C.T. and characterized as a 2-DNA phage with a head and flexible tail without sheath or fibres.

Bacteria were sub-cultured at weekly intervals on to brain heart infusion (BHI) agar, incubated at $37^{0}$C, and kept under anaerobic conditions at all times. Long-term storage was carried out by resuspending cells from 2 day old cultures on BHI agar plates in fresh BHI broth in cryotubes under anaerobic conditions and placing at $-70^{0}$C.

Propagation and storage of high titre phage lysates is described below (2.2.11).

### 2.2.2 MEDIA

BHI broth and agar supplemented with haemin, menadione and cysteine (Holdeman and Moore,1972) were used for bacterial growth at $37^{0}$C.

One-quarter strength Ringer's solution was used as a dilution buffer for cells, and T2 buffer for phage dilutions.

### 2.2.3 ANAEROBIC TECHNIQUES

All anaerobic manipulations were done under stringent anaerobic conditions in an anaerobic glove cabinet (Forma Scientific, Marietta, Ohio, USA) in an atmosphere of 75% $N_2$, 20% $CO_2$, and 5% $H_2$.

All glassware and buffers to be used anaerobically were brought into the cabinet while still warm and allowed to equilibrate for at least 15 h before use.

### 2.2.4 EMS MUTAGENESIS

The optimum concentration and exposure time for EMS mutagenesis of B.fragilis were determined by adding various concentrations of EMS (final concentrations 2.0% and 2.5% v/v) to exponential phase B.fragilis in BHI broth ($A_{600}$ 0.3) and incubating the cultures at $37^0$C. Samples were withdrawn at various time intervals and the number of survivors determined by viable count (Fig. 2.1). The concentration giving approximately 10% survival after 25-30 min EMS exposure was selected for mutation of the cells (Van Tassel and Wilkins, 1978).

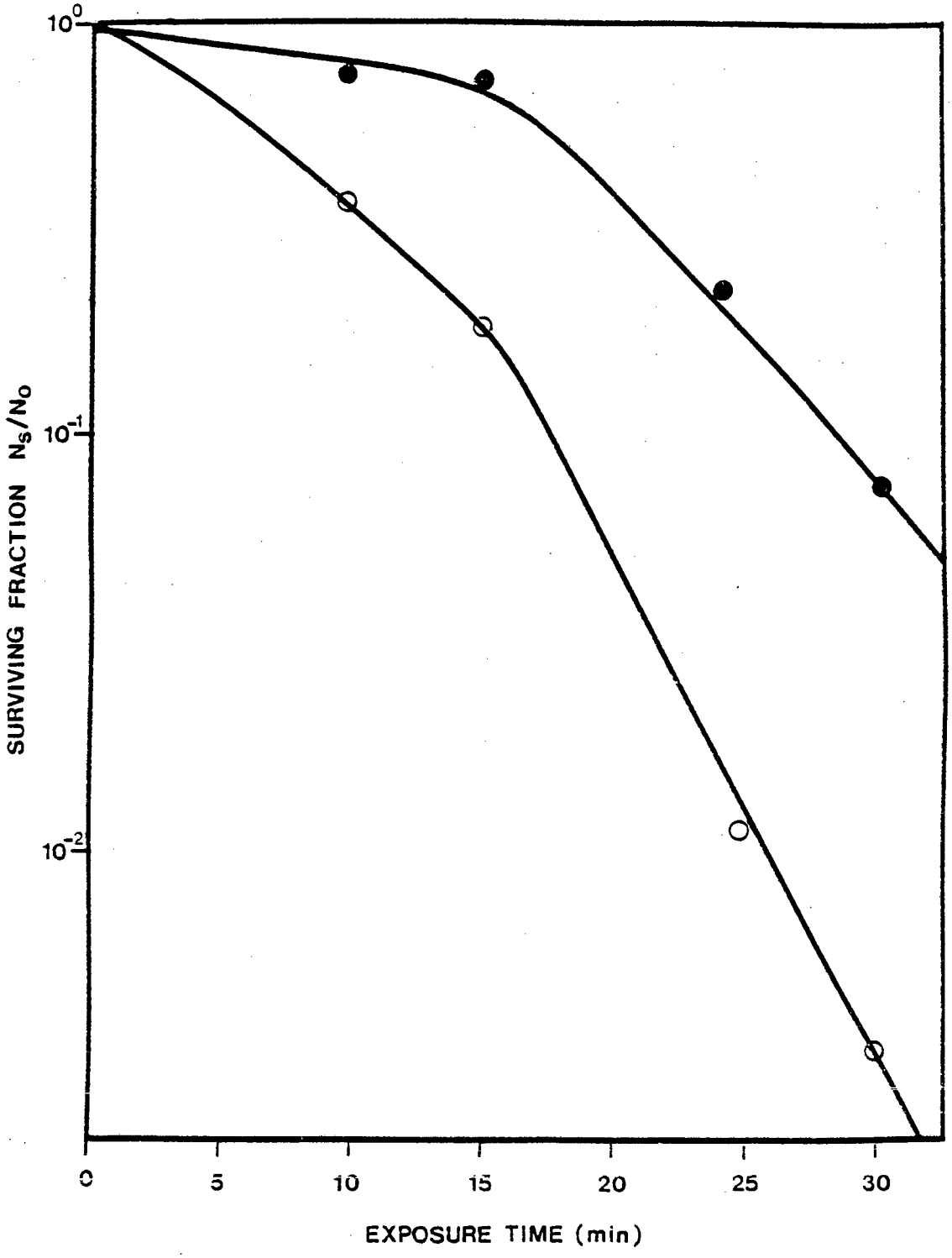



FIGURE 2.1: Survival curves of B.fragilis wild-type cells treated with ethyl methane sulphonate (EMS) and sampled at various time intervals. (●), 2.0% EMS; (○), 2.5% EMS.

2.2.5 ACTIVITY OF MITOMYCIN C UNDER ANAEROBIC CONDITIONS

Four sets of BHI agar plates were made up containing a range of final mitomycin C concentrations from 0-10 $\mu g\ ml^{-1}$ (w/v). The minimum inhibitory concentration (MIC) for B.fragilis Bf-2 cells (100 colony forming units per plate) was determined on freshly-poured plates under aerobic and anaerobic conditions, and on plates which had been stored for 24 h in either an aerobic or anaerobic atmosphere.

2.2.6 ISOLATION OF MUTANTS.

EMS mutagenised cells were sedimented by centrifugation, resuspended in fresh BHI broth and incubated for 20 h at $37^0C$. The culture was diluted and plated to give approximately 100 colony forming units (cfu) $ml^{-1}$. After 18-20 h incubation the pin-prick-sized colonies were replica plated on to agar plates containing mitomycin C (0.1 $\mu g\ ml^{-1}$) and control plates without mitomycin C. Colonies which did not grow on the mitomycin C plates were re-tested for true mitomycin C sensitivity as opposed to low titre effects. Serial dilutions of overnight cultures of the putative mutants were prepared in the wells of a microtitre tray. These were transferred on to a BHI agar control plate and a BHI agar plate containing 0.1 $\mu g\ ml^{-1}$ mitomycin C using a multiple transfer inoculation template (Figs 2.2, 2.3). Cultures which showed true sensitivity

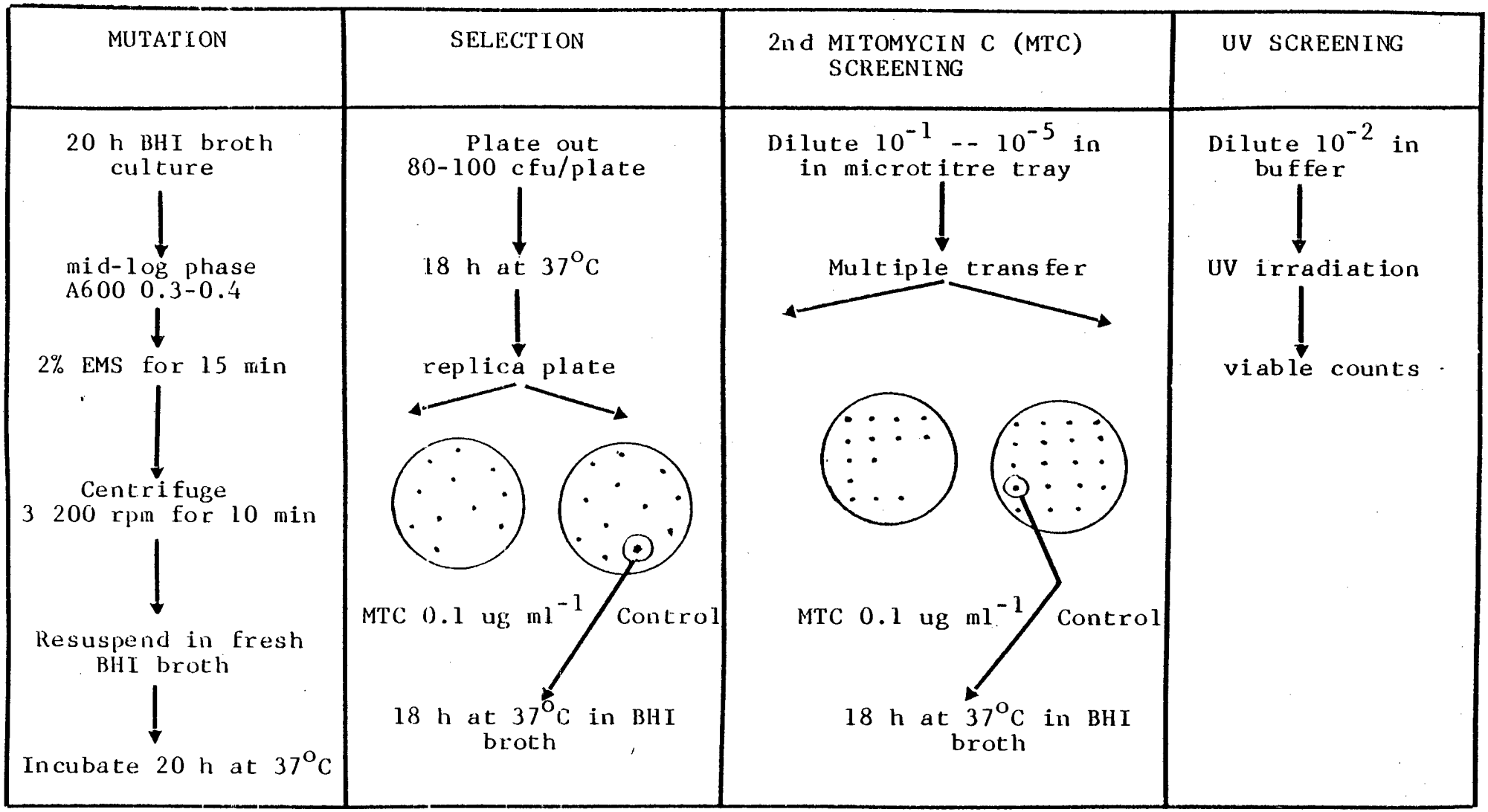



FIGURE 2.2: Protocol for mutagenesis and selection of B.fragilis repair deficient mutants.

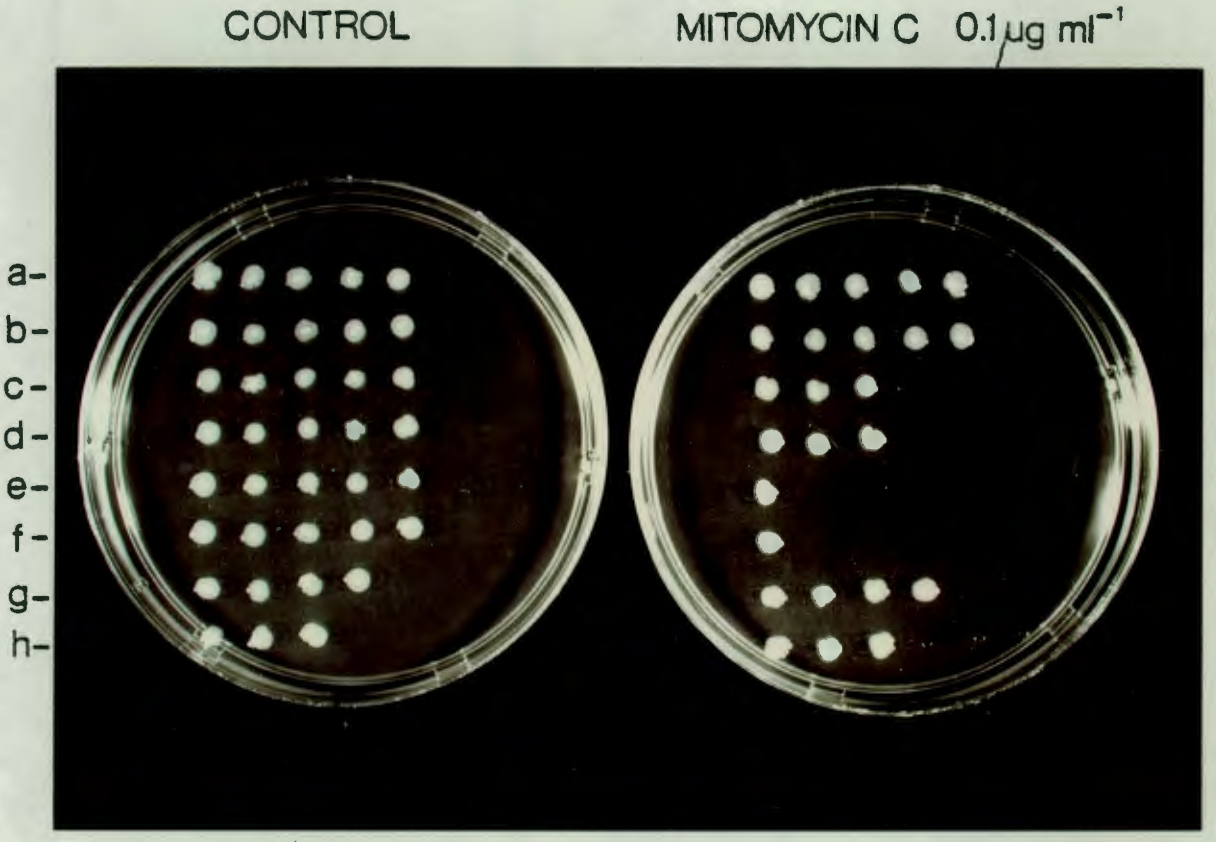



FIGURE 2.3: Second screening for mitomycin C sensitivity using a multiple transfer template.

(a,b) - false positives from replica plating.

(c,d) - intermediate mitomycin C-sensitive strain.

(e,f) - mitomycin C-sensitive strain.

(g,h) - false positives due to low titre.

in the second mitomycin C screening were then tested for UV sensitivity.

2.2.7 UV IRRADIATION

Overnight cultures were diluted $10^{-2}$ into one-quarter strength Ringer's solution to give approximately $1x10^{7}$ cfu $ml^{-1}$. Samples (5 ml) were irradiated in open glass petri dishes with agitation. The source of UV was a Fluotest Piccolo Hanau quartz lamp which emitted the majority of its output at 254 nm. The dose rate was measured with a Blak-Ray UV meter (Model J-225; UV Products Inc., San Gabriel, California). A fluence rate of 1.0 J $m^{-2}$ $s^{-1}$ was used in all experiments.

2.2.8 EFFECT OF MITOMYCIN C AND METHYL METHANE SULPHONATE.

Stationary phase cultures were diluted $10^{-2}$ into anaerobic one-quarter strength Ringer's solution containing either mitomycin C (20 $\mu g$ $ml^{-1}$) or MMS (0.5% v/v) and the number of cfu surviving determined as described below (2.2.9)

2.2.9 MEASUREMENT OF CELL SURVIVAL AFTER VARIOUS TREATMENTS

Survival curves of cells which had been irradiated with UV, or treated with mitomycin C or MMS were determined by removing samples at various time intervals and plating suitable dilutions on BHI agar followed by anaerobic incubation. Cell suspensions which were irradiated aerobically were diluted and plated in air followed by anaerobic incubation.

For experiments to determine the effect of caffeine on UV irradiated cell survival, 1 mg $ml^{-1}$ caffeine was added to the BHI plates.

2.2.10 LIQUID HOLDING RECOVERY

Jones and Woods (1981) optimized the conditions under which liquid holding recovery is observed in B.fragilis Bf-2. The following protocol is based on these findings. Cells were irradiated to 1% survival (30 J $m^{-2}$ for the wild-type and the mitomycin C-sensitive mutant, and 20 J $m^{-2}$ for the UV-sensitive mutant). They were then held at 30°C for 2 h in one-quarter strength Ringer's solution (aerobic or anaerobic), and diluted and plated as described in 2.2.9.

Control unirradiated cells were treated in the same manner.

2.2.11 BACTERIOPHAGE TECHNIQUES AND REACTIVATION OF UV IRRADIATED PHAGE

High titre phage lysates were prepared from the stock suspension by means of confluent lysis on BHI plates using the soft agar overlay technique of Adams (1959). Samples (0.2 ml) of a B.fragilis overnight culture were mixed with 0.1 ml volumes of phage suspension, added to 3 ml soft BHI agar and each mix overlaid on to a BHI agar plate.

The plates were incubated anaerobically overnight at 37°C, after which the plates were each flooded with

5 ml T2 buffer and the soft agar scraped off. The pooled agar suspension was vortexed and allowed to stand for 2 h at $4^0$C, and then centrifuged at 10,000 x g for 20 min to remove the agar. The supernatant phage suspension was stored at $4^0$C over 10% (v/v) chloroform. The number of plaque-forming units (pfu) per ml was determined by making suitable dilutions of the phage suspension in T2 buffer and plating using the soft agar overlay technique.

UV irradiation of the high titre phage suspension was carried out according to the method of Slade et al. (1983a,b). A high titre suspension was diluted at least 100-fold in anaerobic T2 buffer to give a final phage concentration of $10^8$ pfu $ml^{-1}$ prior to irradiation. Volumes (5 ml) of phage suspensions were irradiated in open glass petri dishes with a fluence of 300 J $m^{-2}$. Samples were plated anaerobically with 0.2 ml of overnight cultures of wild-type and mutant strains as described above.

UV reactivation of UV irradiated phage was carried out by exposing mid-log phase ($A_{600}$ 0.4) B.fragilis cells in BHI broth to 120 J $m^{-2}$ and incubating them at $37^0$C for 15 min before infecting with irradiated phage and assaying for pfu $ml^{-1}$. This dose was confirmed as the optimum for the UV-sensitive mutant by exposing the cells to increasing fluences of UV radiation before phage infection of this strain.

$H_2O_2$-induced reactivation was carried out by pre-treating mid-log phase ($A_{600}$ 0.4) *B.fragilis* wild-type and mutant cells with a sub-lethal concentration (0.0025%) of $H_2O_2$ for 5 min at $37^0$C before infecting with UV-irradiated phage and assaying for pfu $ml^{-1}$.

All phage experiments were carried out under stringent anaerobic conditions. Control experiments to determine the efficiency of plating of unirradiated phage on each of the strains tested were carried out together with each experiment.

## 2.3 RESULTS

### 2.3.1 ACTIVITY OF MITOMYCIN C UNDER ANAEROBIC CONDITIONS

The MIC for B.fragilis cells (100 cfu per plate) plated on freshly poured plates either aerobically or anaerobically, or on plates stored aerobically for 24 h before plating and incubation under anaerobic conditions was 0.14 μg mitomycin C $ml^{-1}$. If plates containing mitomycin C were stored anaerobically for 24 h the compound lost activity and the MIC was greater than 7 μg $ml^{-1}$ mitomycin C (Table 2.1).

### 2.3.2 ISOLATION OF MITOMYCIN C- AND UV-SENSITIVE MUTANTS.

The mutagenesis and screening protocol carried out (Figs 2.1, 2.2 and 2.3) yielded 25 putative mitomycin C-sensitive mutants at the first mitomycin C screening stage. The second screening eliminated false positives by differentiating between lower titre effects and true mitomycin C sensitivity. Six mutants were identified as being sensitive to mitomycin C. Five of these showed similar sensitivity to mitomycin C (MIC 0.06 μg $ml^{-1}$ on plates) (Table 2.2) and they had wild-type resistance to UV irradiation. These strains may have been siblings since they were all isolated from the same mutagenic experiment, and one of them (MTC25) was chosen for further study.

TABLE 2.1:

Effect of anaerobic conditions on mitomycin C activity.

Brain heart infusion agar plates containing various concentrations of mitomycin C were plated with cells (100 c.f.u.) either immediately or after 24 h storage under anaerobic or aerobic conditions.

| Mitomycin C µg ml-1 | Storage Conditions | | |
|---|---|---|---|
| | Aerobic/Anaerobic Immediate use | Aerobic 24 h | Anaerobic 24 h |
| 0.0 | ++ | ++ | ++ |
| 0.1 | + | + | ++ |
| 0.5 | - | - | ++ |
| 1.0 | - | - | ++ |
| 3.0 | - | - | ++ |
| 5.0 | - | - | ++ |
| 7.0 | - | - | ++ |
| 10.0 | - | - | - |

++ 100 c.f.u. per plate
+ 1 - 9 c.f.u. per plate
- No growth

TABLE 2.2

Repair phenotypes of wild-type *B. fragilis* and mutant strains.

| Strain | [a] F 0.37 ($J\ m^{-2}$) | [b] Mitomycin $C_1$ MIC ($\mu g\ ml^{-1}$) |
|---|---|---|
| Wild-type | 15 | 0.14 |
| MTC25 | 15 | 0.06 |
| UVS9 | 10 | 0.08 |

[a] Mean lethal dose (dose resulting in 37% survival of cells).

[b] MIC - minimum inhibitory concentration on plates.

Growth of the sixth mitomycin-sensitive mutant was inhibited on plates by 0.08 $\mu g\ ml^{-1}$ mitomycin C, a concentration intermediate between that which inhibited the wild-type (0.14 $\mu g\ ml^{-1}$) and MTC25 (0.06 $\mu g\ ml^{-1}$) strains. This mutant, designated UVS9, showed increased sensitivity to UV irradiation. All three strains had the same level of sensitivity to MMS. Representative survival curves showing sensitivity to UV radiation, mitomycin C and MMS are shown in Figs 2.4, 2.5 and 2.6 respectively. The mean UV radiation lethal dose (Harm, 1980) for the wild-type and MTC25 strains was 15 J $m^{-2}$ (Table 2.2), and the relative sensitivity of the UVS9 mutant was 1.5-fold.

### 2.3.3 EFFECT OF CAFFEINE ON SURVIVAL AFTER UV IRRADIATION

The MIC of caffeine for the wild-type, MTC25 and UVS9 strains was 2.5 mg $ml^{-1}$. When these three strains were irradiated and plated on BHI agar containing 1 mg $ml^{-1}$ caffeine, a marked decrease in survival was observed compared with the survival on the control plates (Fig. 2.4.). The dose reduction factor (Harm, 1980) at 1% survival was 0.7 for each of the three strains. Unirradiated cells showed no decrease in survival when plated on this concentration of caffeine-BHI agar.

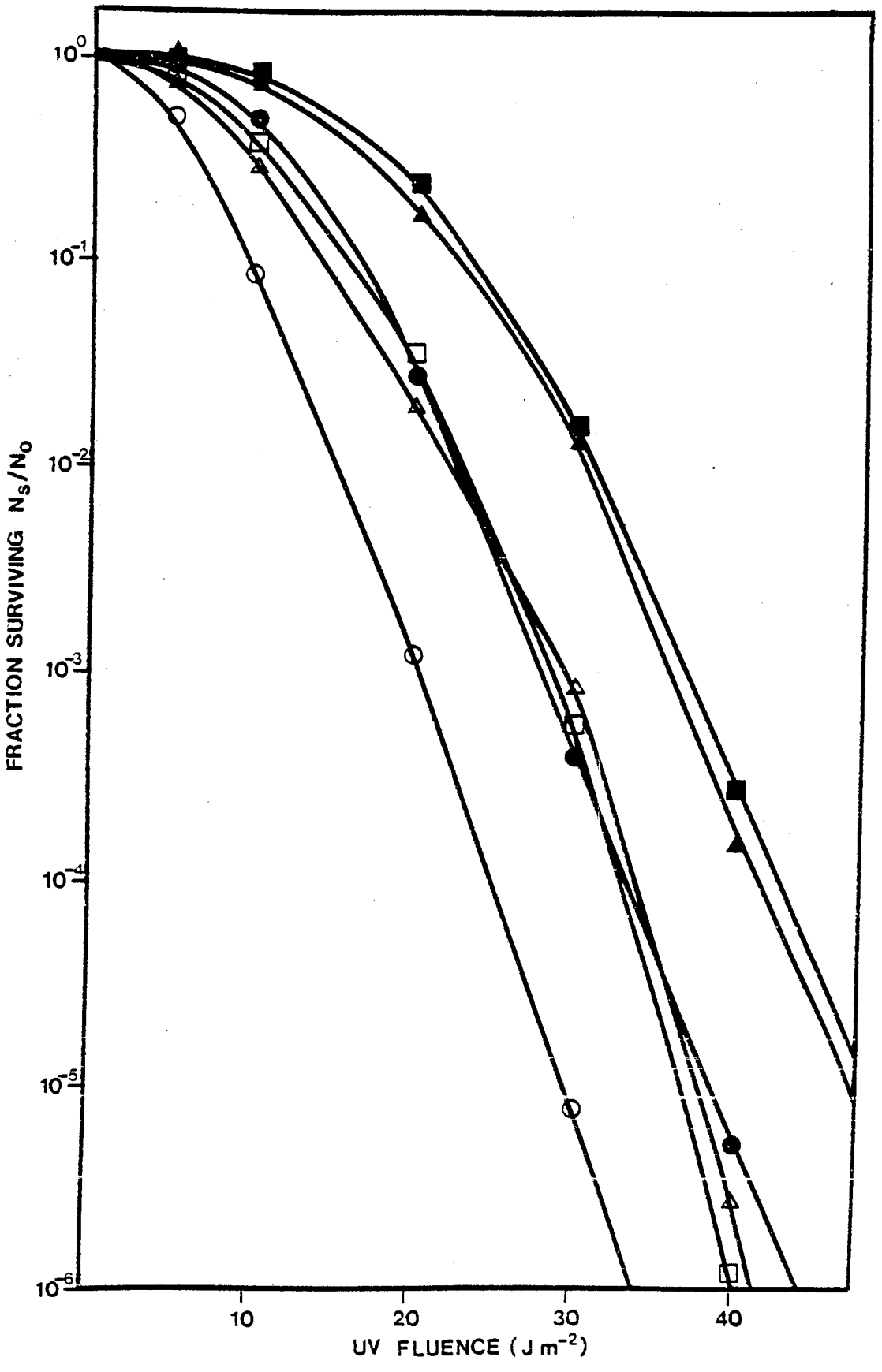



FIGURE 2.4: Effect of caffeine (1 mg $ml^{-1}$) on the survival of UV-irradiated B.fragilis strains.

Wild-type plated with (□) and without (■) caffeine;

MTC25 with (△) and without (▲) caffeine;

UVS9 with (○) and without (●) caffeine.

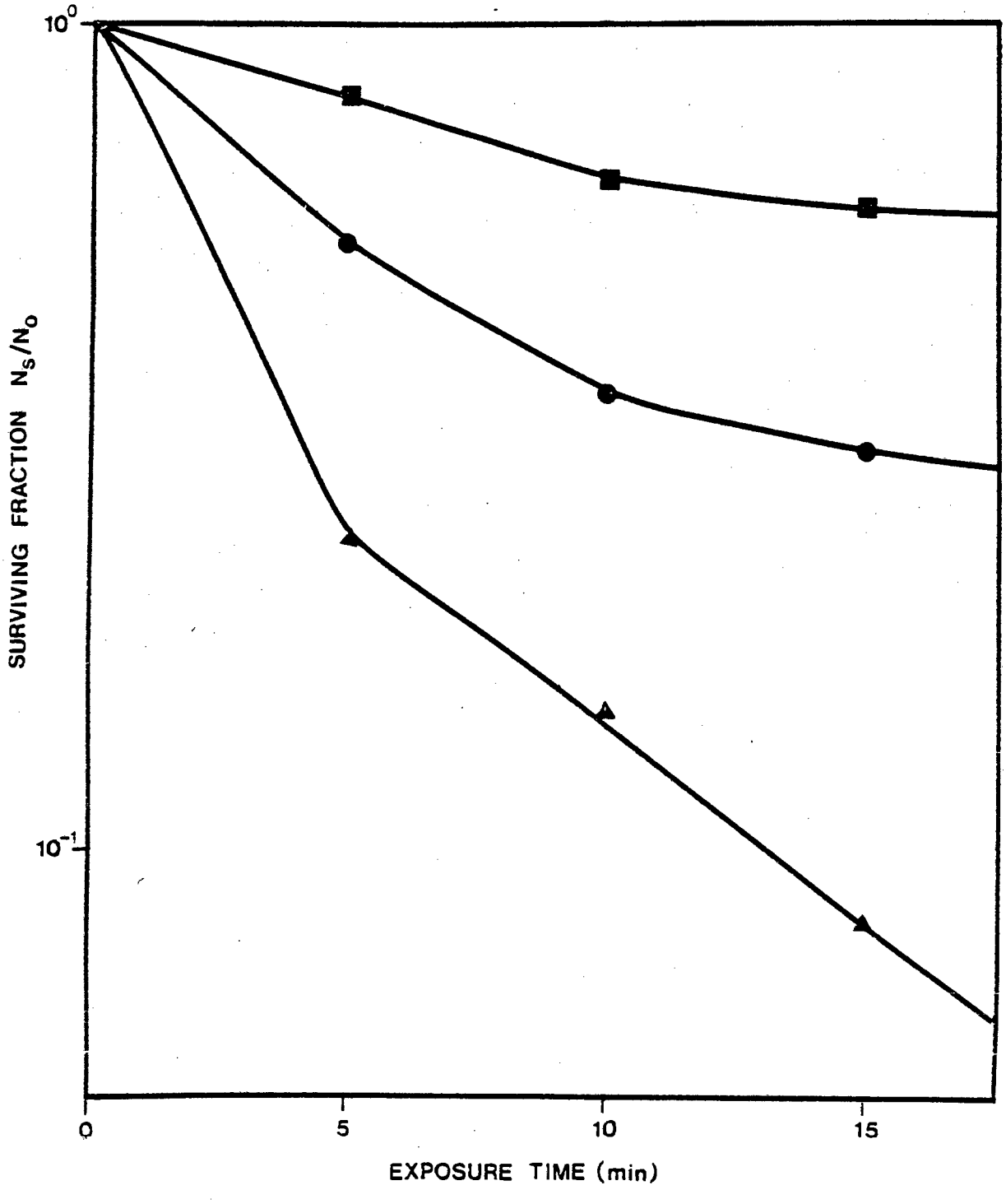



FIGURE 2.5: Effect of mitomycin C on the survival of B.fragilis strains. Stationary phase cultures were diluted $10^{-2}$ into anaerobic one-quarter-strength Ringer's solution containing 20 µg mitomycin C $ml^{-1}$ and the number of cfu determined. ■, Wild-type strain; ▲, strain MTC25; ○, strain UVS9.

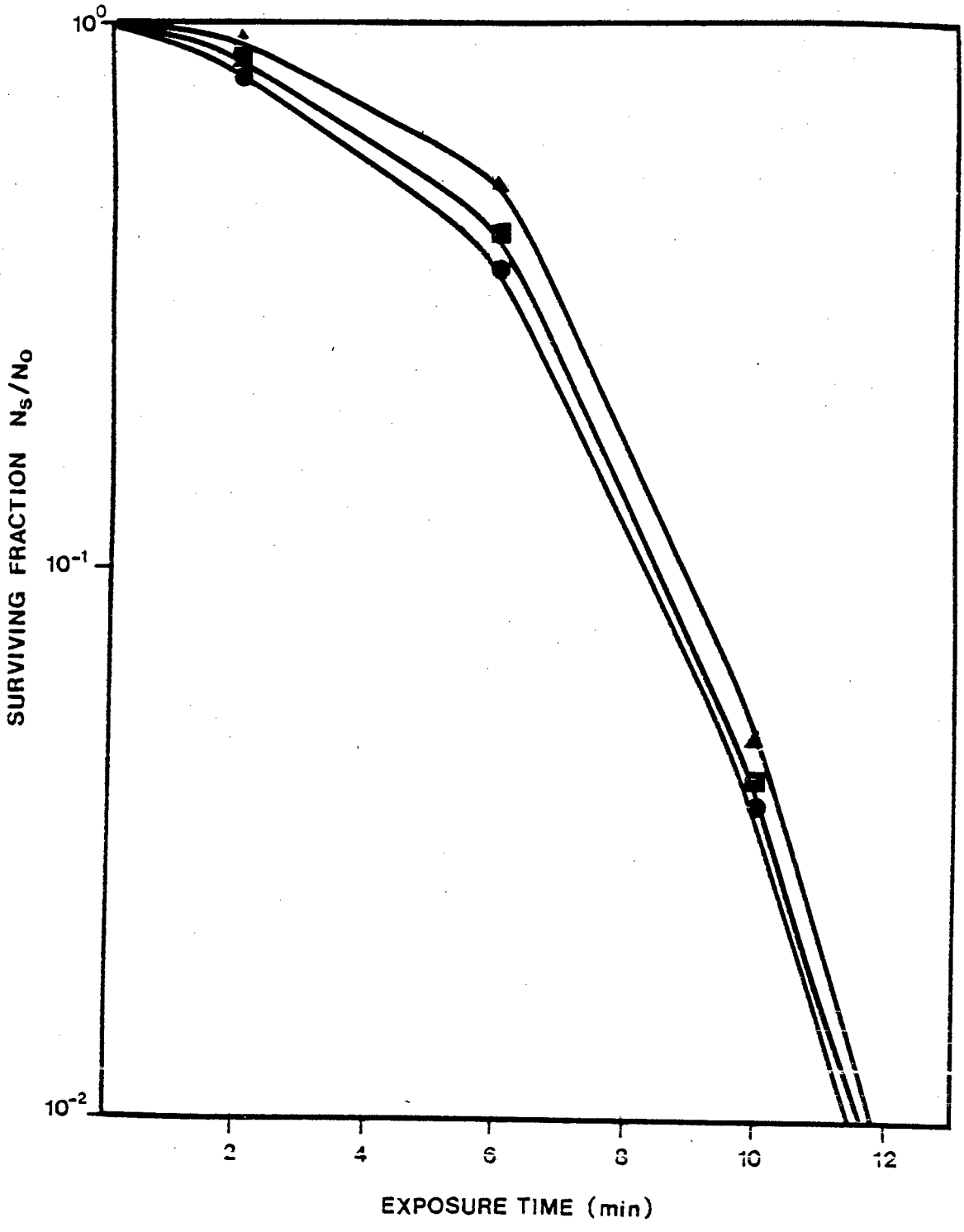



FIGURE 2.6: Effect of methyl methane sulphonate (MMS) on the survival of B.fragilis strains. Stationary phase cultures were diluted $10^{-2}$ into anaerobic one-quarter-strength Ringer's solution containing 0.5% (v/v) MMS and the number of cfu determined.

■, Wild-type; ▲, MTC25; ●, UVS9.

2.3.4 LIQUID HOLDING RECOVERY

The survival of irradiated stationary and exponential phase wild-type, MTC25, and UVS9 cells was enhanced after holding in aerobic one-quarter strength Ringer's solution at $30^0$C (Table 2.3). The liquid holding recovery after 2 h holding was approximately 2.5-fold greater in irradiated exponential than stationary phase cells. Unirradiated cells held under the same conditions showed no change in survival. Irradiated stationary phase wild-type and MTC25 strains held under anaerobic conditions showed a decrease in survival whereas the UVS9 strain showed a small increase in survival. Unirradiated cells held anaerobically showed no change over the holding period.

2.3.5 BACTERIOPHAGE REACTIVATION

Slade et al. (1983a,b) investigated the optimum conditions for UV- and $H_2O_2$-induced phage reactivation in B.fragilis Bf-2. Similar experiments with mutants MTC25 and UVS9 indicated that the optimum conditions for UV irradiation and $H_2O_2$ treatment were the same as for the wild-type. The efficiency of plating of the unirradiated phage on the wild-type, MTC25 and UVS9 strains was identical.

The MTC25 and UVS9 mutants both showed a decrease in uninduced host cell phage reactivation relative to the wild-type (Fig. 2.7A), and there was also a lower level of UV-induced reactivation than that found in the wild-type. $H_2O_2$-induced reactivation was, however, 33% lower in the UVS9 mutant than that found in the wild-type and MTC25 mutant strains (Fig. 2.7B)

| STRAIN | STATIONARY | | EXPONENTIAL |
|---|---|---|---|
| | AEROBIC | ANAEROBIC | AEROBIC |
| WILD-TYPE | 2.1 | 0.45 | 5.1 |
| MTC25 | 1.8 | 0.48 | 4.9 |
| UVS9 | 2.3 | 1.6 | 6.2 |

Table 2.3: Liquid Holding Recovery.
Stationary and exponential phase cells were irradiated to 1% survival and held at 30°C for 2 h in anaerobic or aerobic one-quarter-strength Ringer's solution. Results are expressed relative to irradiated cells plated immediately. Data from the average of five independent experiments are given. The results from all the experiments were within +/- 9 - 10% of the values given.

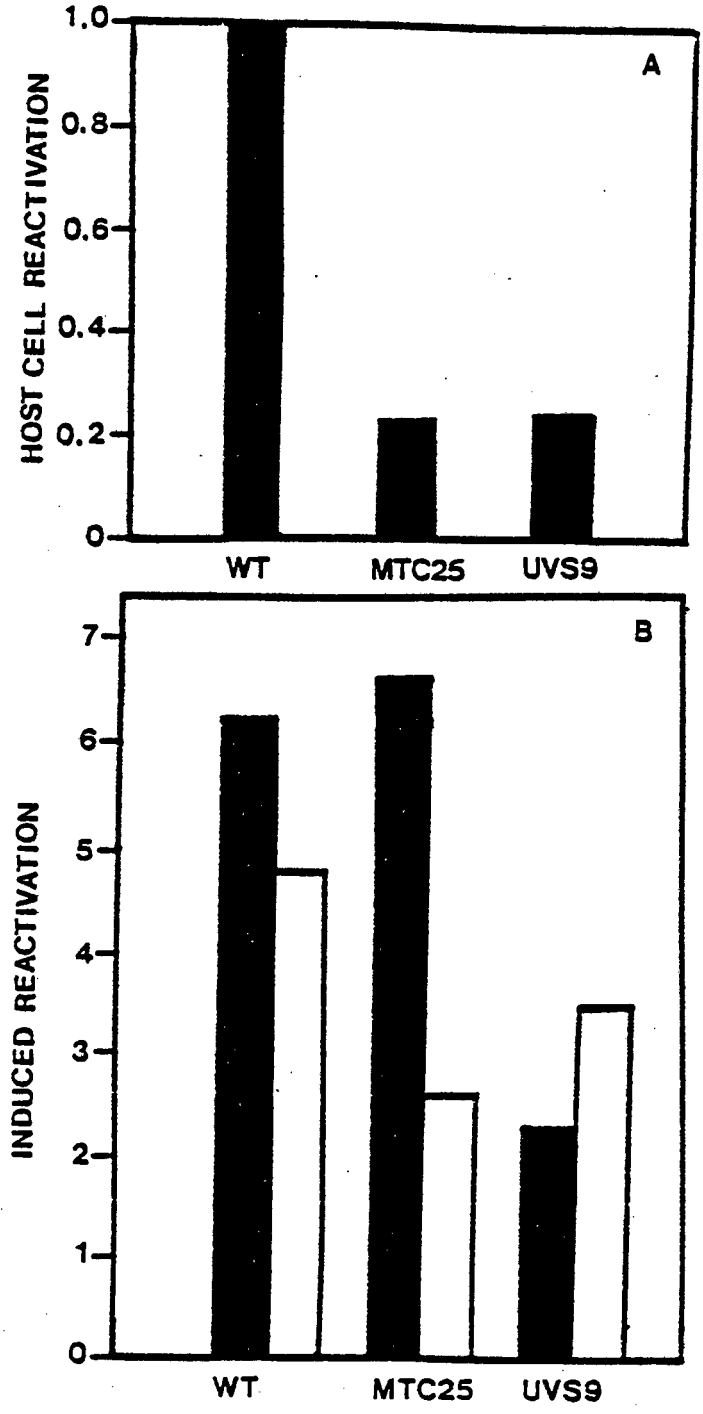
FIGURE 2.7: Bacteriophage Reactivation.

Phage b-1 was UV-irradiated (300 J $m^{-2}$), and the extent of

A: uninduced host cell reactivation and

B: ( □ ) UV-, and ( ■ ) $H_2O_2$-induced phage reactivation

were determined as described in the text.




A: The levels of the two mutant strains are expressed relative to the wild-type expressed as 1. The mean of eight experiments is shown.

B : Results are expressed relative to the uninduced level of that strain taken as 1. The mean of six and four expriments is shown for $H_2O_2$ and UV, respectively.

## 2.4 DISCUSSION

The activity of mitomycin C has been reported to be affected by anaerobic conditions (Szybalski and Iyer, 1967), and was shown in this study to be inactivated in BHI agar after 24 h in the anaerobic cabinet. It was nevertheless successfully used for the isolation of two different mitomycin C-sensitive B.fragilis mutants by the use of freshly-poured plates under controlled experimental conditions.

The MTC25 mutant is markedly sensitive to mitomycin C, but is unaffected as regards UV sensitivity. The UVS9 mutant is sensitive to UV but is only moderately sensitive to mitomycin C. A similar correlation between the repair of mitomycin C- and UV-induced lesions has been observed in M.radiodurans (Moseley and Copland, 1978) and has been discussed in Section 2.1.

The fact that all three of the strains tested had the same level of sensitivity to MMS indicates that a recA-like gene is probably not involved in the observed UV/mitomycin C-sensitivity of the mutants.

The B.fragilis UVS9 mutant showed a dose reduction factor (Harm, 1980) of 0.6 at 1% survival compared to the wild-type. The main difference between the UV survival curves of the wild-type and UVS9 strains was in the shoulder region where repair of the DNA occurs at

low UV doses. The shoulder region is still present though reduced in the UVS9 survival curve indicating some residual repair either via a leaky mutation or via some other cellular repair system. These two possibilities have been further examined in Chapters III and IV.

Caffeine has been found to reduce cell survival after UV irradiation in several organisms including E.coli (Sideropoulos et al., 1968), S.typhimurium (Williams and Clarke, 1971), and N.gonorrhoeae (Campbell and Yasbin, 1984a). Notable exceptions to this trend are two strains of M.radiodurans which are very resistant to radiation damage (Sweet and Moseley, 1974). These are thought to have very accurate repair mechanisms which are not caffeine sensitive.

Although it has been widely used in DNA repair studies as an inhibitor of excision repair, (reviewed by Timson, 1977), the exact nature of the action of caffeine has only recently been elucidated. Seeberg and Strike (1976) showed that caffeine inhibited the action of the pyrimidine dimer endonuclease in E.coli, and Rothman (1980) found that it inhibited the rate at which endonucleolytic incisions were made, and also prolonged the time required to rejoin the discontinuities. Fong and Bockrath (1979) also reported an inhibition of incision in the presence of caffeine.

Most of the work on caffeine has been carried out in E.coli, and equivalent studies in other bacterial systems are still at an early stage. However, Campbell and Yasbin (1984a) have found that the presence of caffeine in the holding buffer of UV irradiated cells inhibited the removal of pyrimidine dimers by N.gonorrhoeae.

In this study, caffeine was found to decrease the survival of irradiated B.fragilis wild-type and mutant strains. The possibility of it acting on a pyrimidine dimer excision system in B.fragilis was explored in Chapters III and IV.

In E.coli liquid holding recovery is observed in cells having functional uvr and polA gene products, but only if the cells have some other decreased repair capacity as is found in E.coli B or in E.coli K12 recA mutants (Ganesan and Smith, 1968, 1969). In B.fragilis, aerobic liquid holding recovery occurs in the wild-type as well as both mutant strains. Under anaerobic conditions where the wild-type is functioning optimally, a negative liquid holding effect was observed. This is a phenomenon usually found in cells which are repair proficient and where holding is detrimental to recovery (Harm, 1980). The MTC25 mutant also showed a negative holding effect, which is in agreement with the wild-type level of UV resistance found in this strain. The UV-sensitive UVS9 strain however, showed a tendency towards recovery on holding

under anaerobic conditions which correlates with its UV-sensitive phenotype. Jones and Woods (1981) reported the inhibition of liquid holding recovery under anaerobic conditions but did not observe the negative liquid holding effect.

$H_2O_2$-induced phage reactivation revealed a further difference between the two B.fragilis mutant strains. Phage reactivation in the MTC25 mutant was induced to the same extent as in the wild-type by $H_2O_2$, but the UVS9 mutant showed reduced $H_2O_2$-induced phage reactivation.

The development of protocols for the isolation and physiological characterization of the first repair deficient mutants of an anaerobic bacterium lays the foundation for further work in this direction and for genetic studies on B.fragilis at the molecular level.

# CHAPTER III

## PYRIMIDINE DIMER EXCISION REPAIR IN BACTEROIDES FRAGILIS: ALKALINE AGAROSE GEL ELECTROPHORESIS ANALYSIS.

## SUMMARY

An enzyme preparation purified from Micrococcus luteus was shown to be specific for UV-induced pyrimidine dimers and was suitable for the detection of DNA excision repair systems. The wild-type B.fragilis Bf-2 strain and a mitomycin C-sensitive mutant (MTC25) had constitutive dimer excision systems which functioned efficiently under anaerobic and aerobic conditions. A UV-sensitive mutant (UVS9) had markedly reduced levels of the constitutive dimer excision system under anaerobic and aerobic conditions. Caffeine (2.5 mg $ml^{-1}$) completely inhibited dimer excision in the wild-type strain under anaerobic conditions while having no lethal effect on unirradiated cells. Holding irradiated cells in caffeine (2.5 mg $ml^{-1}$) before plating on caffeine-free medium resulted in decreased survival compared to irradiated cells held in buffer only. Unirradiated cells showed no loss in viability during holding in caffeine. The final level of excision of dimers was unaffected by the presence of chloramphenicol under both aerobic and anaerobic conditions although the rate of removal was halved. Since liquid holding recovery under aerobic conditions is completely inhibited by chloramphenicol, it is concluded that pyrimidine dimer removal is not the process responsible for increased physiological aerobic liquid holding recovery.

## 3.1 INTRODUCTION

Studies of DNA repair mechanisms of E.coli and several other prokaryotic and eukaryotic cell systems have shown that the cellular responses to DNA damage fall broadly into three major categories viz: The reversal, the excision, and the tolerance of the damage (Chapter IC).

The molecular analysis of DNA repair mechanisms in UV-irradiated bacteria thus depends largely on the assay of pyrimidine-containing dimers formed as a result of the radiation treatment, and their removal or tolerance in the DNA at various stages of the repair process.

Several procedures have been developed for the quantitative separation of thymine-containing dimers from hydrolysed, UV-irradiated, radiolabelled DNA. These include thin-layer chromatography (Cook and Friedberg, 1976; Reynolds et al., 1981), paper chromatography (Carrier and Setlow, 1971; Carrier, 1981), ion-exchange chromatography (Varghese and Wang, 1967; Sekiguchi and Shimizu, 1981), and high-performance liquid chromatography (HPLC) (Breter et al., 1974; Love and Friedberg, 1982). All of these procedures depend on a high degree of radiolabelling of the DNA, and, except for the HPLC technique of Love and Friedberg (1982), are not sensitive enough to detect very low, yet biologically significant, levels of thymine dimers.

An alternative approach to the detection of pyrimidine dimers is by means of a UV dimer-specific enzyme probe. Two such enzymes have been identified to date. They are the phage T4 endonuclease V, the product of the Den V gene of T4 (Yasuda and Sekiguchi, 1970; Friedberg and King, 1971; Wood and Ravel, 1976), and the UV-specific endonuclease isolated from M.luteus (Carrier and Setlow, 1970; Riazuddin and Grossman, 1977a,b). Both these enzymes have pyrimidine dimer DNA glycosylase activity which cleaves the glycosylic bond between the 5'-pyrimidine of the dimer and its corresponding sugar, generating an apyrimidinic site. In addition, they possess an associated apurinic/apyrimidinic endonuclease activity which cleaves the phosphodiester bond on the 3' side of the base-free sugar (Fig. 3.1) (Haseltine et al., 1980; Gordon and Haseltine., 1980, 1981). The nicks generated in radiolabelled, double-stranded, UV-irradiated DNA treated with a dimer specific enzyme probe may be visualized and quantified by sedimentation through alkaline sucrose gradients (Wilkins, 1973; Ganesan et al., 1981), or by nitrocellulose membrane filtration (Braun, 1981).

An alternative method using alkaline agarose gel electrophoresis was devised by Campbell and Yasbin (1984a) for visualizing nicks in unlabelled UV-irradiated DNA following UV endonuclease treatment.

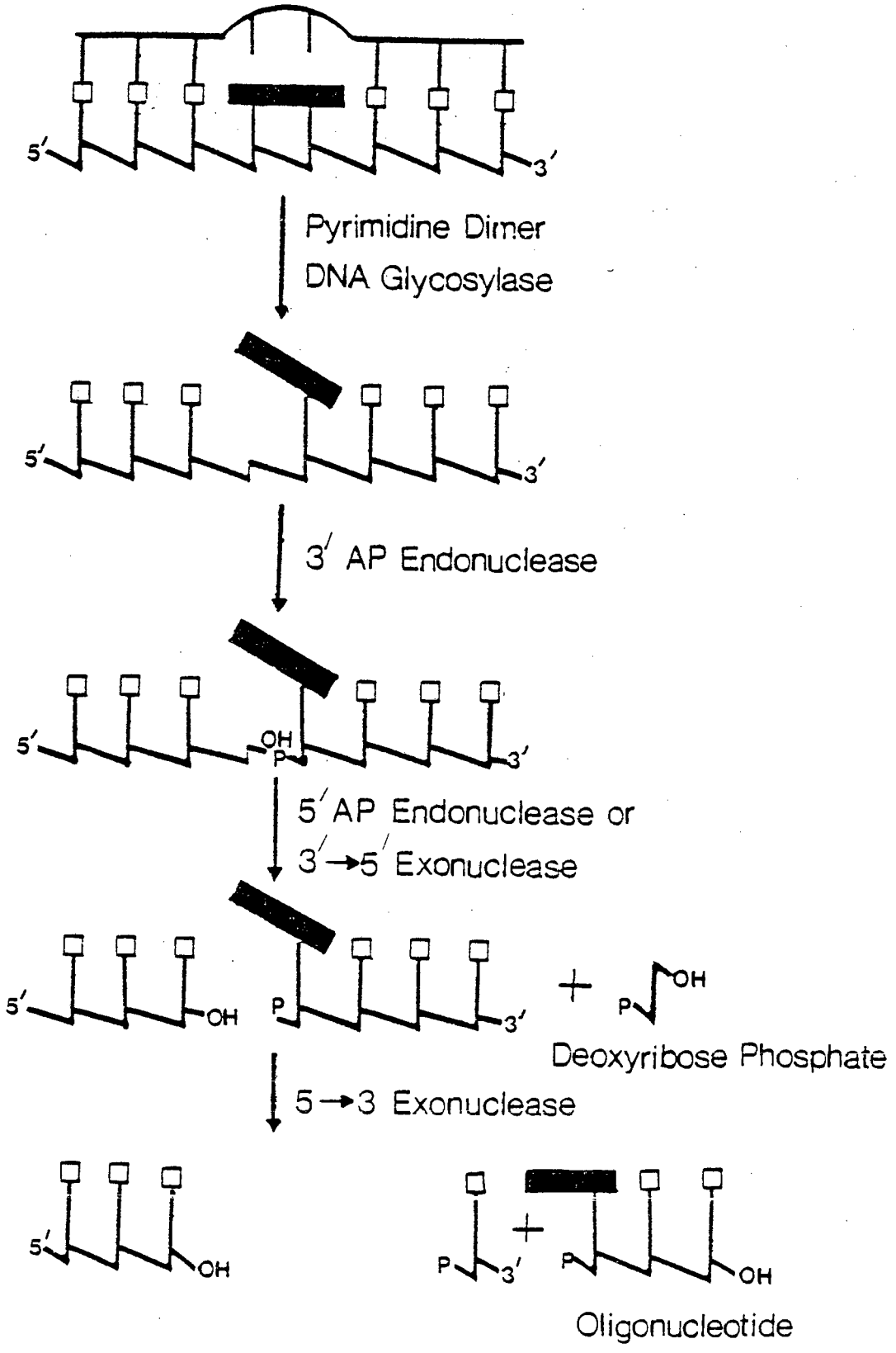



FIGURE 3.1: Mode of action of the pyrimidine dimer specific glycosylase/endonuclease enzyme.

This method was thought to be suitable for studying the excision of pyrimidine dimers from UV-irradiated B.fragilis cells, for, although B.fragilis is able to incorporate exogenous radiolabelled thymidine into DNA, the level of incorporation is generally insufficient to allow excision repair studies involving radiolabelled dimers at low UV doses. Jones and Woods (1981) used radiolabelled B.fragilis DNA and thin layer chromatographic analysis to detect the excision of thymine dimers from irradiated DNA during aerobic and anaerobic holding in buffer. However, Jones (1979) reported that overloading of the chromatograms in an attempt to obtain higher thymine dimer counts was a draw-back of this technique.

In view of the difficulties associated with labelling and the complexities of this method compared to the Campbell and Yasbin (1984a) method, the latter was considered more suitable.

It should be noted here that the object of these studies was a semi-quantitative assessment of the persistence or removal of pyrimidine dimers in wild-type B.fragilis and its UV-/mitomycin C-sensitive mutants. The potential for extending the methodology to allow for highly accurate quantitation of dimers is discussed in 3.4.

## 3.2 METHODS

### 3.2.1 BACTERIA

The *B.fragilis* wild-type strain (Bf-2), and mitomycin C-sensitive (MTC25) and UV-sensitive (UVS9) mutants of this strain, were utilised; they have been described in 2.2.1.

### 3.2.2 MEDIA

Difco BHI broth and agar, one-quarter-strength Ringer's solution dilution buffer, and stringent anaerobic conditions were used as described in 2.2.2.

### 3.2.3 UV IRRADIATION

Methods for irradiating cells under aerobic and anaerobic conditions were as described in 2.2.3.

### 3.2.4 PURIFICATION OF UV ENDONUCLEASE FROM M.LUTEUS.

This was done by a modification of the methods of Grafstrom *et al*. (1982) and Hays *et al*. (1985). Unless otherwise stated, all procedures were carried out at 4°C. The crude *M.luteus* cell-free extract was prepared from spray-dried cell powder of the ATCC strain 4698 (Miles Chemical Co., Elkhart, Ind.). Two 25 g samples of the spray-dried cells were suspended in 25 ml ice-cold buffer (20 mM-Tris/HCl, pH 8.00), centrifuged at 8,000 x g for 10 min at 4°C, and washed once in the same buffer. Each pellet was rigorously resuspended in 37.5 ml of Buffer A (20 mM-Tris/HCl, pH 8.00; 1 mM -Na-EDTA; 1 mM -2-mercaptoethanol; 10% glycerol) at room temperature

and the samples pooled. The cells were lysed by the addition of lysozyme to a final concentration of 1 mg $ml^{-1}$ (75 mg lysozyme) and incubated at 37°C for 15 min. The suspension was quickly chilled to 0°C by adding 25 ml frozen Buffer A and holding in an ice-ethanol bath. The following protease inhibitors were added to the final concentrations indicated: 0.5 ml of 1 M-$NaHSO_3$ (final concentration 5 mM), and 0.05 ml of 0.5 M phenylmethylsulphonate (final concentration 0.25 mM). The cells were sonicated at maximum output in chilled 10 ml volumes using 5 x 30 sec bursts until the viscosity disappeared. The combined homogenates were centrifuged at 48,000 x g for 30 min, and the supernatant brought to 0.3 M-NaCl by the dropwise addition of 5 M-NaCl. After stirring for 30 min at 4°C, the the suspension was centrifuged at 48,000 x g for 30 min and the supernatant retained (Fraction A of Grafstrom et al., 1982).

The crude M.luteus extract was loaded onto an Affi-Gel Blue (Biorad) column, bed volume 50 ml, equilibrated with four column volumes of 0.3 M-NaCl in buffer A. The column was washed stepwise with this buffer, first containing 0.3 M-NaCl (100 ml) , and then 1.2 M-NaCl (400 ml); the enzyme activity was then eluted with 3 M-NaCl in approximately 300 ml. The column flow rate throughout was maintained at 5-10 ml $h^{-1}$. The NaCl was removed by dialysis against 2 x 10 vol of the elution buffer containing no NaCl, and the partially purified

endonuclease precipitated with 70% ammonium sulphate with 45 min stirring at 4°C. After centrifugation at 30,000 x g for 15 min, the protein pellet was resuspended in storage buffer (50 mM-HEPES/NaOH, pH 7.6, containing 100 mg bovine serum albumin $ml^{-1}$, 1 mM-dithiothreitol, 0.19% (v/v) Triton X-100 and 50% (v/v) glycerol), and dialysed overnight against 2 x 100 vol of the same buffer to remove all salts. Storage was at -20°C.

### 3.2.5 PROTEIN ASSAY

The protein concentration of the endonuclease preparation was measured according to the micro-protein method of Bradford (1976).

### 3.2.6 SEMI-QUANTITATIVE ASSAY OF UV ENDONUCLEASE ACTIVITY

The concentrated enzyme preparation was diluted 1:100 and 1 µl added to 340 ng (supercoiled) pBR322 DNA, in endonuclease buffer (3.2.7), which had been irradiated with a dose of 150 J $m^{-2}$. After 1 h digestion at 37°C, the mix was electrophoresed on a 0.8% agarose gel, stained with ethidium bromide and viewed for conversion to relaxed open circular plasmid DNA.

### 3.2.7 DNA EXCISION REPAIR

Overnight BHI broth cultures of the *B.fragilis* strains were diluted 10-fold into 10 ml pre-warmed BHI broth and grown to mid-exponential phase ($A_{600}$ 0.3-0.4). The cells were harvested anaerobically by centrifugation and resuspended in 10 ml one-quarter-strength aerobic or

anaerobic Ringer's solution. The cell suspensions were exposed to a UV dose of 15 J $m^{-2}$ as described in 2.2.7. Samples were removed either immediately after irradiation or after post-irradiation incubation at 30°C.

In experiments to determine the effect of chloramphenicol (Cm) on dimer excision, 10 µg Cm $ml^{-1}$ was added to growing cells 15 min before irradiation and/or to the holding buffer after irradiation. The effect of caffeine on the removal of dimers was determined by adding 0.25, 0.5, or 2.5 mg caffeine $ml^{-1}$ to the holding buffer under anaerobic conditions.

Cells from 1 ml samples were harvested by centrifugation, resuspended in 0.5 ml cold lysis buffer (50 mM -Tris/HCl, pH 8.0; 10 mM -Na-EDTA; 500 mM -NaCl), and lysed at 4°C for 10-15 min by the addition of SDS to a final concentration of 1%. The DNA was extracted with phenol and diethyl ether, and precipitated with 99% (v/v) ethanol during overnight storage at -20°C. The precipitate was pelleted by centrifugation and resuspended in 100 µl endonuclease buffer (20 mM-Tris/HCl; 40 mM-NaCl; 2 mM-EDTA, pH 8.0).

Repair of UV damage was assayed as follows:-
The UV endonuclease was diluted 1:100 and 2 µl was added to 20 µl DNA. After 1 h incubation at 37°C, 5 µl alkaline tracking dye (see Appendix) was added and the entire sample was electrophoresed on a horizontal, 0.5% alkaline

agarose gel (Appendix) for 20-24 h at 16-20 volts. After electrophoresis, the gel was neutralised for at least 30 min in 0.1 M-Tris, pH 8.0, and then stained for 30 min in 0.1 M-Tris, pH 8.0 containing 0.5 µg ethidium bromide $ml^{-1}$. The gel was photographed using Polaroid negative film and quantification was done by scanning the negative with a Beckman DU 8 spectrophotometer (Wood, 1985; Freeman et al., 1986). The amount of high molecular weight DNA present after the various treatments was related to undigested control samples included in each gel by peak triangulation.

## 3.3 RESULTS

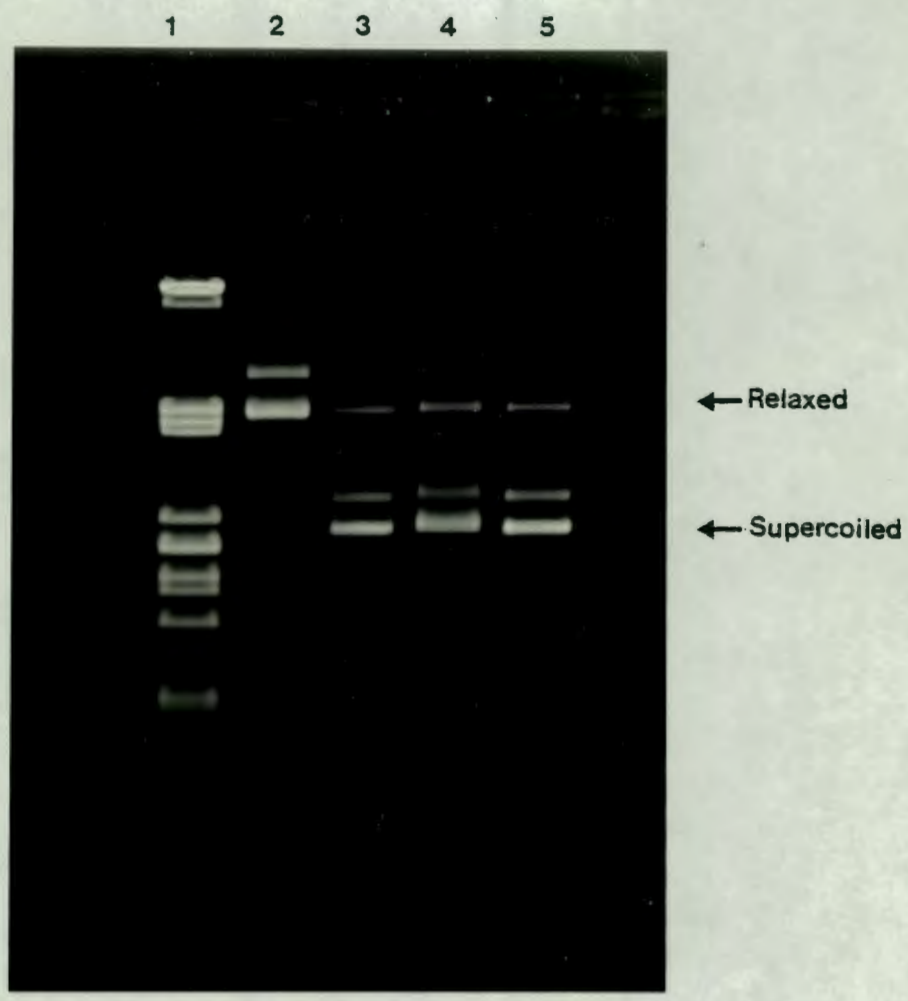
### 3.3.1 PREPARATION OF THE M.LUTEUS DIMER SPECIFIC ENDONUCLEASE.

The enzyme prepared as described was specific for UV-irradiated pBR322 DNA when assayed semi-quantitatively (Hays et al., 1985). A 1 µl sample of a 1:100 dilution of the concentrated preparation of the enzyme (0.1 µg protein; Bradford, 1976) completely relaxed 340 ng supercoiled pBR322 DNA irradiated with a dose of 150 J $m^2$ (Fig. 3.2). The enzyme specifically nicked irradiated B.fragilis chromosomal DNA; this was detected on alkaline agarose gels as a broad smear of small DNA fragments which sometimes masked the plasmid(s) contained in the Bf-2 cells (Fig. 3.3A). The enzyme had no effect on unirradiated B.fragilis DNA (Fig. 3.3A) and the chromosomal DNA appeared as a zone of high molecular mass DNA with the small plasmid clearly visible. Holding of unirradiated cells in buffer for up to 2 h caused no degradation of the chromosomal DNA (Fig. 3.3B).

### 3.3.2 EXCISION OF PYRIMIDINE DIMERS

The wild-type B.fragilis strain was able to remove pyrimidine dimers during holding in buffer under anaerobic and aerobic conditions (Figs 3.5A, 3.6A, 3.7A). The time course for the removal of dimers over a 1 h period under anaerobic conditions is shown in Fig. 3.4.

FIGURE 3.2: Semi-quantitative assay of M.luteus pyrimidine dimer specific endonuclease activity on UV-irradiated pBR325 supercoiled DNA.
340 ng supercoiled pBR325 DNA (irradiated, 150 J $m^{-2}$, or unirradiated control DNA) were digested at 37°C for 1 h and electrophoresed on 0.8% agarose gels (TBE buffer).

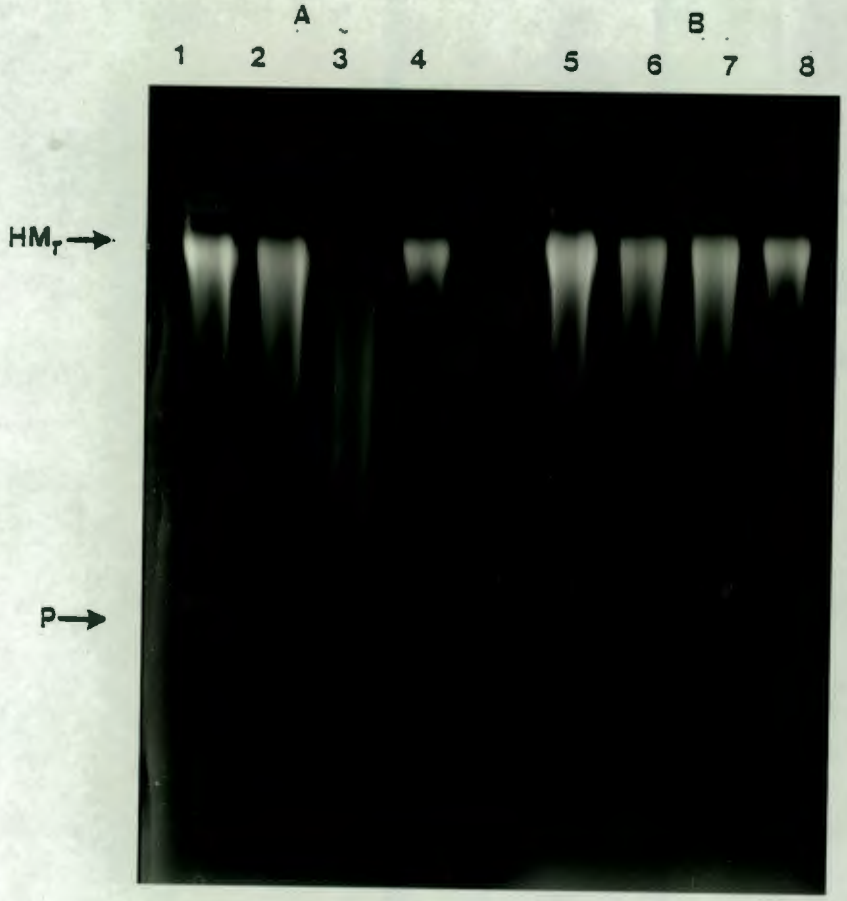



Lane 1: λ PstI DNA control
2: UV-irradiated pBR325 DNA + endonuclease
3: UV-irradiated pBR325 DNA
4: Unirradiated pBR325 DNA + endonuclease
5: Unirradiated pBR325 DNA

FIGURE 3.3: Effect of the dimer specific M.luteus UV endonuclease on irradiated (15 J $m^{-2}$) and unirradiated B.fragilis chromosomal DNA.

$HM_r$ high $M_r$ chromosomal DNA

P plasmid DNA



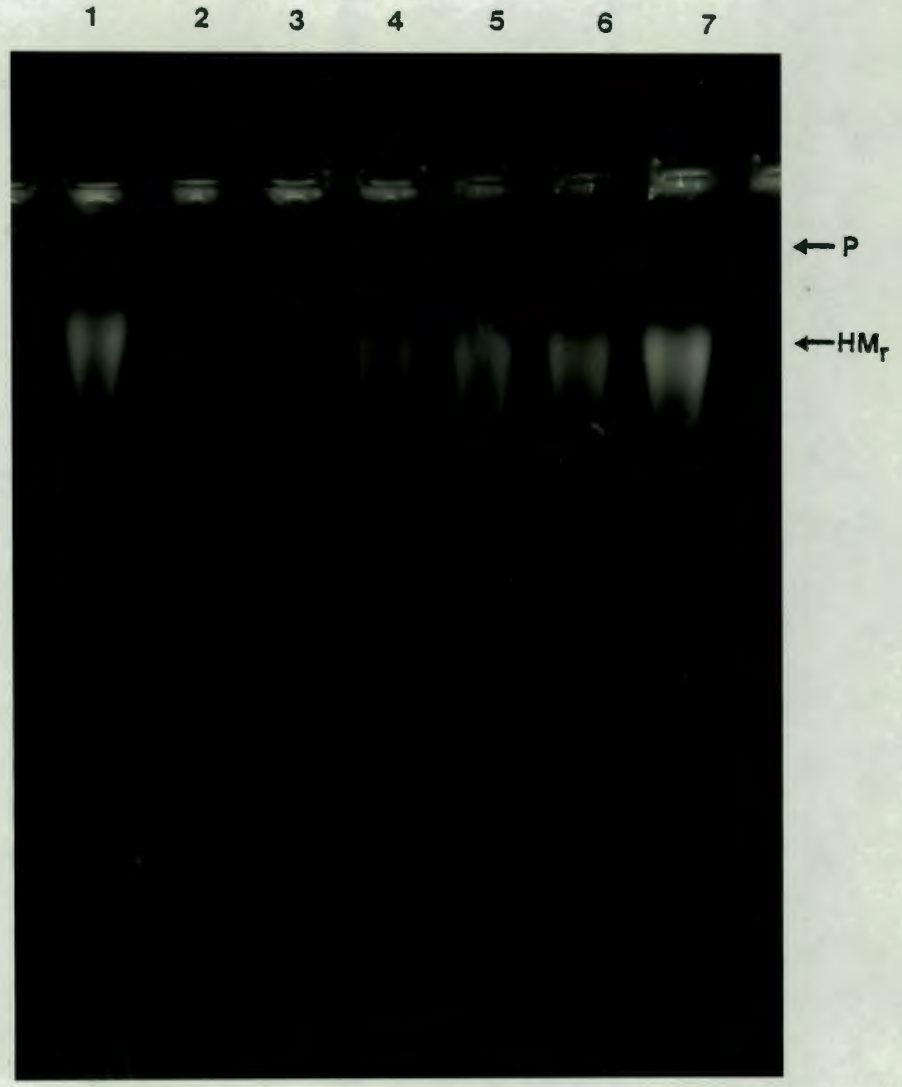

Key:

| Treatment | Lane 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| UV | - | - | + | + | - | - | - | - |
| Endonuclease | + | - | + | - | + | - | + | - |
| Holding time (h) | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |

FIGURE 3.4: The time course of dimer excision by *B.fragilis* wild-type cells (irradiated with 15 J $m^{-2}$) during anaerobic post-irradiation holding at 30°C.

$HM_r$ High $M_r$ chromosomal DNA

P plasmid DNA




Key:

| | Lane | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| UV | + | + | + | + | + | + | + |
| Endonuclease | - | + | + | + | + | + | - |
| Holding time (min) | 0 | 0 | 15 | 30 | 45 | 60 | 60 |

Immediately after irradiation the chromosomal DNA contained pyrimidine dimers and so was nicked by the M.luteus dimer specific endonuclease. After 1 h holding in buffer under aerobic or anaerobic conditions, the dimers had been removed by a cellular repair mechanism and the enzyme-treated DNA was not nicked. Densitometer tracings of the gel photographs clearly reflected the results seen on the gels (Fig. 3.6). The profiles for nicked and repaired DNA could be easily distinguished and the degree of dimer removal estimated as a percentage of the undigested control of each sample (Fig. 3.7). The mitomycin C-sensitive (MTC25) strain also showed dimer removal within 1 h under aerobic and anaerobic conditions (Figs 3.5B, 3.6B, 3.7B). In comparison with the wild-type, the MTC25 strain showed slightly lower levels of dimer removal under anaerobic conditions but the same level aerobically. The UV-sensitive (UVS9) mutant showed very much reduced dimer removal under both aerobic and anaerobic conditions (Figs 3.5C, 3.6C, 3.7C ).

### 3.3.3 EFFECT OF CHLORAMPHENICOL ON DIMER REMOVAL

When 10 μg of Cm $ml^{-1}$ was added to the holding buffer after irradiation there was no noticeable effect on the rate or final levels of dimer removal achieved by any of the strains tested (Fig. 3.7A,B,C.). When the same concentration of Cm was added to the growing cells 15 min prior to irradiation as well as to the holding buffer,

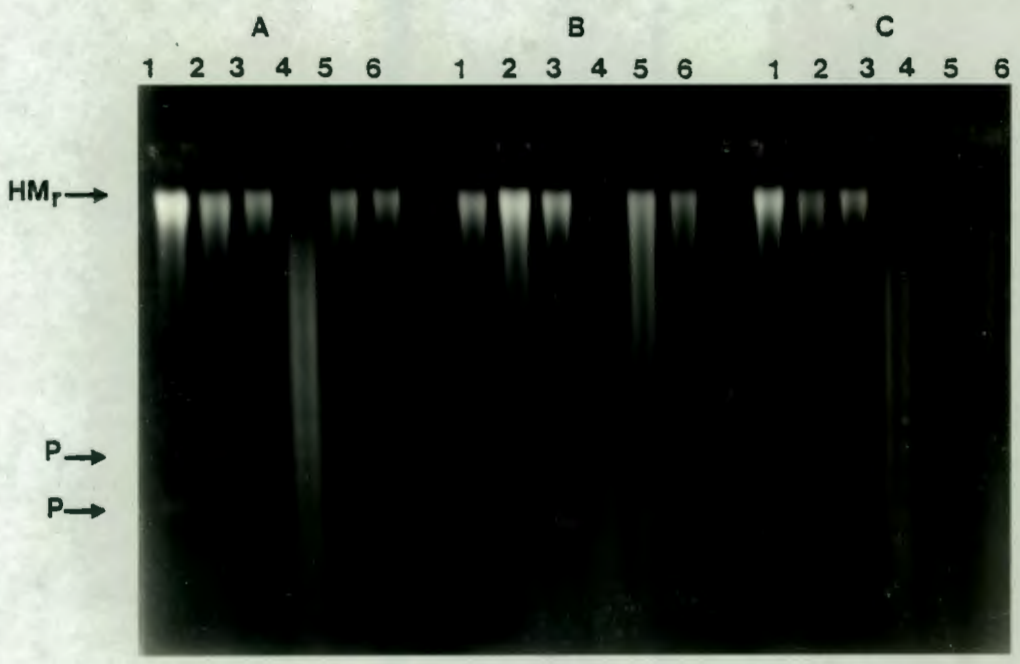
FIGURE 3.5: Dimer excision by B.fragilis Bf-2, MTC25 and UVS9 strains under anaerobic conditions.

Cells were irradiated with UV (15 J $m^{-2}$) and assayed for dimer removal using the M.luteus dimer specific endonuclease during anaerobic holding in buffer at 30°C.

A, wild-type; B, MTC25; C, UVS9.

$HM_r$ High $M_r$ chromosomal DNA

P Plasmid DNA




Key:

| | Lane | | | | | |
|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 |
| UV | + | + | + | + | + | + |
| Endonuclease | - | - | - | + | + | + |
| Holding time (h) | 0 | 1 | 2 | 0 | 1 | 2 |

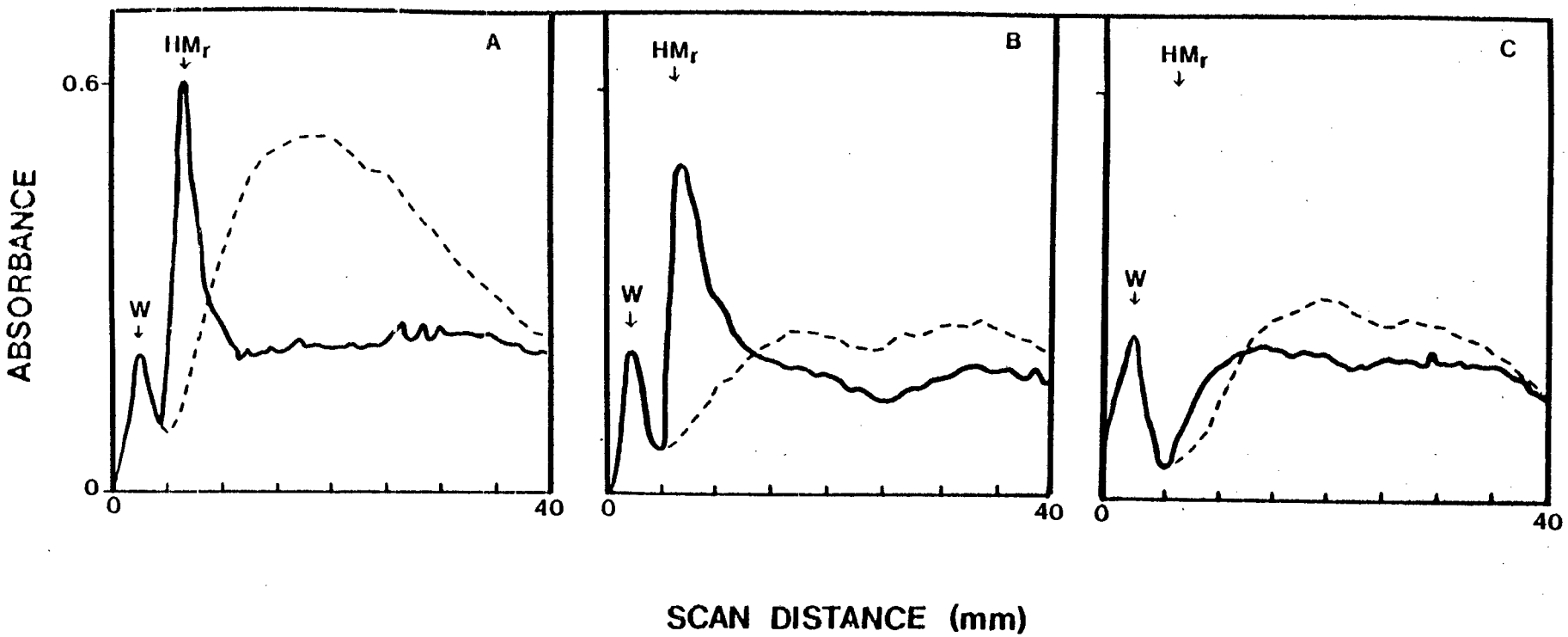



FIGURE 3.6: Densitometer tracings of alkaline agarose gel shown in Fig. 3.5. A, wild-type; B, MTC25; C, UVS9.

- - -, 0 h post-irradiation holding time (lane 4, Fig. 3.5)

———, 2 h post-irradiation holding time (lane 6, Fig. 3.5)

$HM_r$, High $M_r$ chromosomal DNA; W, Gel well.

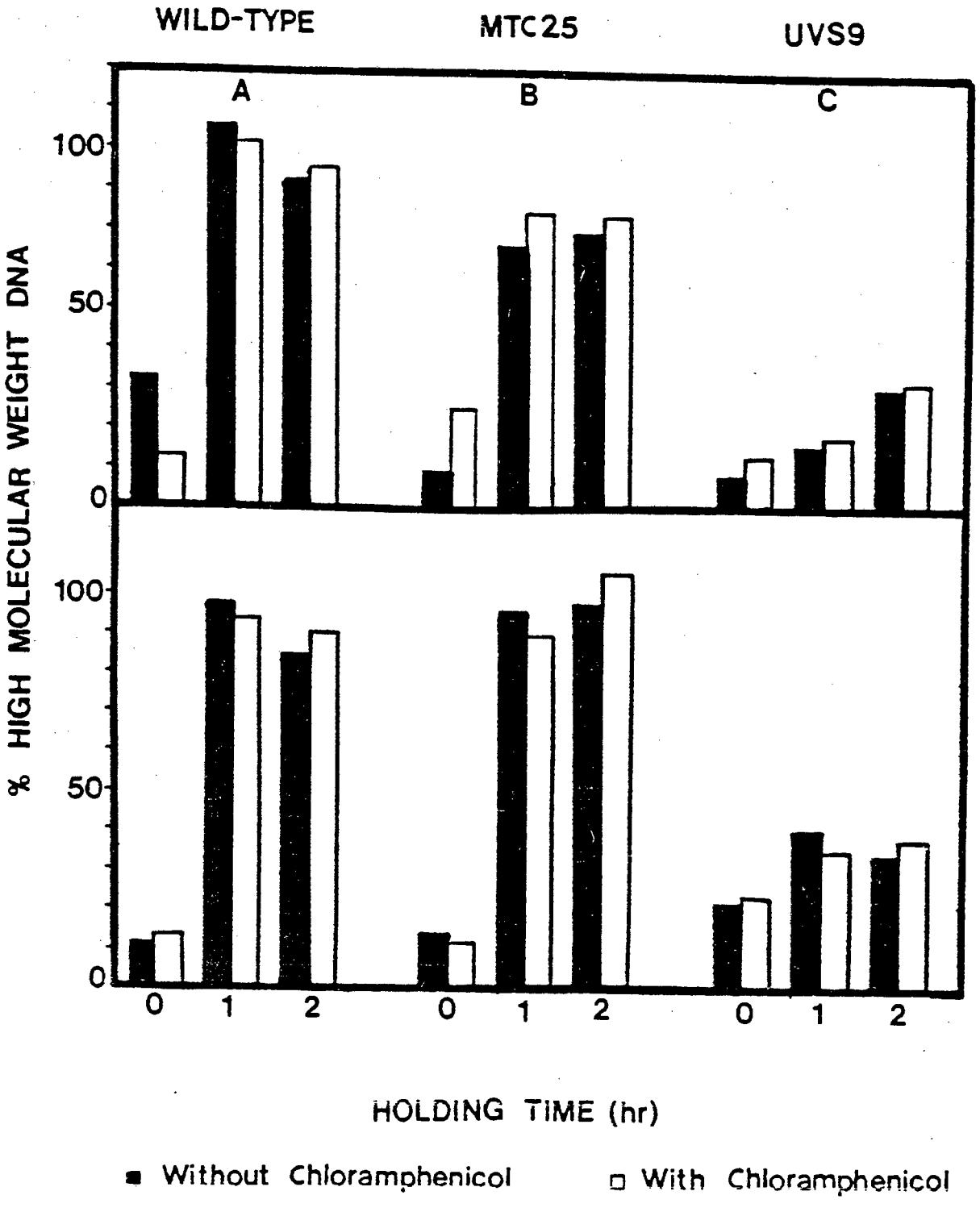



FIGURE 3.7: Effect of post-irradiation holding in chloramphenicol on dimer excision in B.fragilis under aerobic and anaerobic conditions.

Chloramphenicol (10 $\mu g\ ml^{-1}$) was added to the holding buffer during anaerobic or aerobic post-irradiation holding at 30°C. The range of results obtained in four experiments is given.

FIGURE 3.8: Effect of pre- and post-irradiation treatment with chloramphenicol (10 $\mu g\ ml^{-1}$) on the rate and maximum levels of dimer excision in B.fragilis.

Chloramphenicol (10 $\mu g\ ml^{-1}$) was added 15 min before irradiation (15 $J\ m^{-2}$) and during holding in buffer under anaerobic conditions.

A, MTC25; B, wild-type; C, UVS9.

$HM_r$ High $M_r$ chromosomal DNA

P Plasmid DNA


Key:

| | Lane | | | | | |
|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 |
| UV | + | + | + | + | + | + |
| Endonuclease | - | - | - | + | + | + |
| Holding Time (h) | 0 | 1 | 2 | 0 | 1 | 2 |

same maximum level of dimer removal was obtained at 2 h but the rate of removal was approximately halved (Fig. 3.8A,B,C).

### 3.3.4 EFFECT OF CAFFEINE ON DIMER REMOVAL

The addition of 2.5 mg caffeine $ml^{-1}$ to the holding buffer under anaerobic conditions completely inhibited the excision of pyrimidine dimers from the irradiated DNA, even after 2 h holding (Fig. 3.9D). Removal of the cells to a caffeine-free buffer after 1 h exposure, followed by a further 1 h holding period, did not enhance dimer removal (Fig. 3.9E). The inhibitory effect of caffeine on dimer removal was also seen after 1 h holding at a concentration of 0.5 mg caffeine $ml^{-1}$ (Fig. 3.9C), but excision repair was observed after 2 h. Very little inhibiton of dimer removal occurred when 0.25 mg caffeine $ml^{-1}$ was used (Fig. 3.9B).

The viability of the irradiated cells held anaerobically in caffeine (2.5 mg $ml^{-1}$) for 2 h and then plated on caffeine-free medium showed a 2-3 fold decrease compared to irradiated cells held in buffer alone (Fig. 3.10). This concentration of caffeine had no effect on the viability of unirradiated cells.

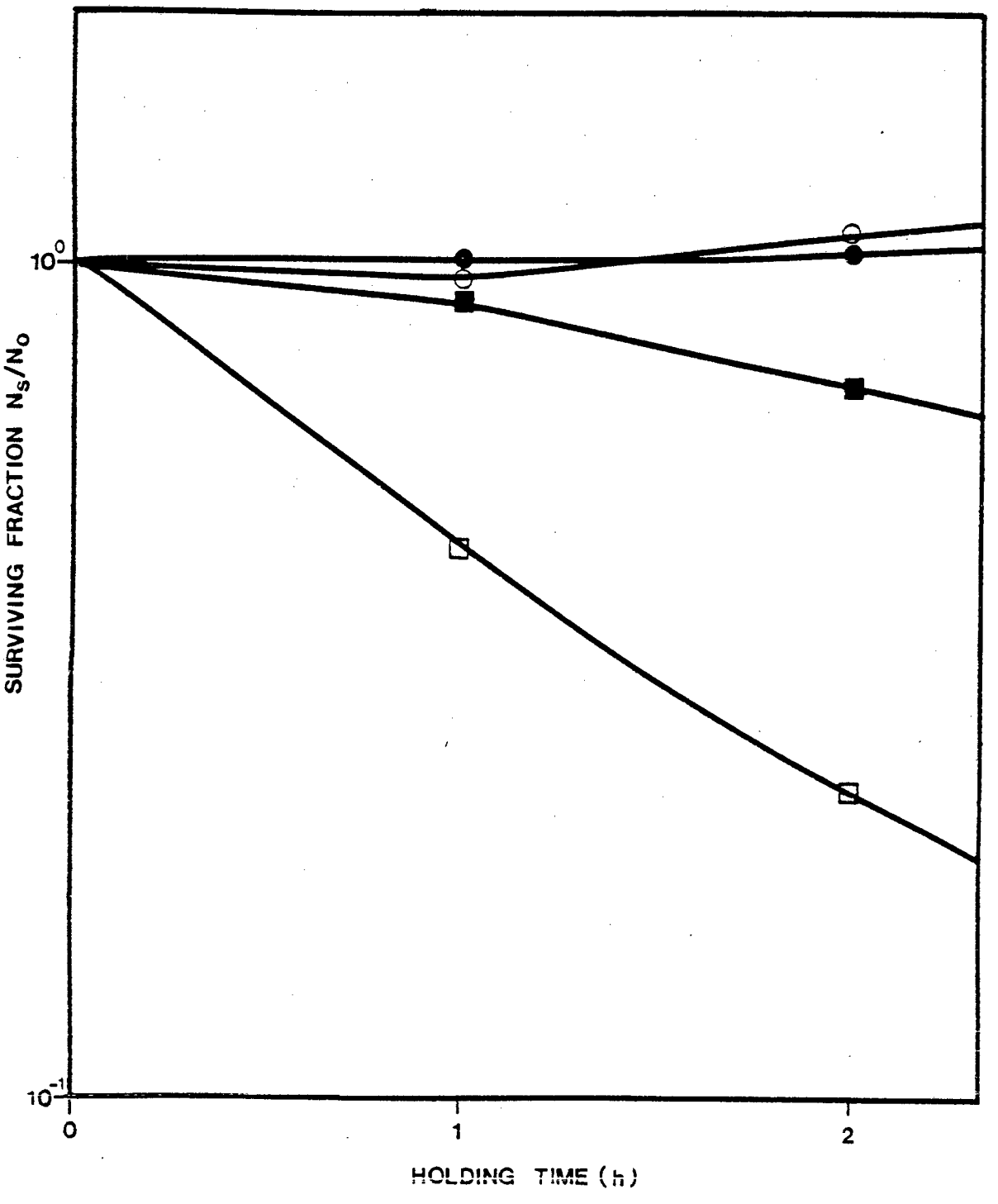



FIGURE 3.10: Survival of irradiated (15 J $m^{-2}$) and unirradiated B.fragilis wild-type cells after anaerobic holding in buffer containing 2.5 mg caffeine $ml^{-1}$ and plating on caffeine-free medium.
Unirradiated cells with ( ○ ), and without ( ● ) caffeine.
Irradiated cells with ( □ ), and without ( ■ ) caffeine.

## 3.4 DISCUSSION

A simplified method of preparation of a crude extract of the pyrimidine dimer specific UV endonuclease from M.luteus was used in this study. The enzyme showed specificity for UV-induced pyrimidine dimers and was suitable for the detection of DNA excision repair systems in irradiated, unlabelled B.fragilis cells. Irradiation of the cells at 15 J $m^{-2}$ was chosen for these experiments as this is a conveniently low dose falling at the end of the shoulder region of the B.fragilis wild-type survival curve (approx 30% survival). Lower doses (5 and 10 J $m^{-2}$) gave similar but less easily visualized results on the gels.

The effect of Cm on the excision of dimers was examined to establish whether the repair system of B.fragilis is constitutive and/or induced. Schumann et al. (1984) showed that a concentration of 10 µg Cm $ml^{-1}$ inhibits protein synthesis in this strain. When Cm was added 15 min prior to irradiation and to the holding buffer, the rate of repair was approximately halved but attained the same maximum level of total repair after 2 h as in untreated cells. Since dimer excision was not abolished by chloramphenicol, it is concluded that there is a constitutive dimer excision system in B.fragilis. The decrease in the rate of dimer excision in cells pre-treated with Cm could be due either to the effect of

Cm on the physiological state of the cells, or to the inhibition of synthesis of another induced dimer excision system which enhanced the rate of repair. A constitutive and inducible dimer excision system of this type has been reported to function in E.coli (Chapter I, Section C) in the regulation of the uvrA,B,C genes.

Jones et al. (1981) reported that liquid holding recovery occurred in B.fragilis Bf-2 only under aerobic conditions and that it was completely inhibited by adding 10 $\mu$g Cm $ml^{-1}$ to the holding buffer immediately after irradiation (Jones, 1979). The results reported here show that dimer excision was not inhibited under these experimental conditions. Removal of pyrimidine dimers from irradiated B.fragilis DNA during aerobic holding was therefore not the process responsible for increased physiological recovery under these conditions. This observation is supported by the fact that dimer excision was found to occur under anaerobic conditions where no physiological liquid holding recovery was observed (Jones et al., 1981). Furthermore, the UV-sensitive mutant showed very low levels of dimer excision under aerobic conditions (20-40%), while maintaining the same level of physiological liquid holding recovery as the wild-type (Abratt et al., 1985). This result differs from that found in E.coli K12 recA mutants where dimer recovery is

essential for liquid holding recovery (Ganesan and Smith, 1969).

The mitomycin C-sensitive mutant (MTC25) showed slightly reduced levels of dimer removal compared with the wild-type strain under anaerobic conditions (50-70%) in spite of having wild-type resistance to UV. This suggests that dimer removal is not he only repair system functional in B.fragilis.

The UV-sensitive mutant was not totally deficient in dimer removal. It retained a low level of dimer excision and had a residual shoulder (indicating repair) in the UV survival curve at low UV doses (Abratt et al., 1985). The low level of repair in the UVS9 mutant could be interpreted either as evidence for a second repair system or, alternatively, that the mutant has a leaky mutation and that excision of dimers is only reduced.

The effect of caffeine on UV-irradiated cells has been reviewed in Section 2.4. The work reported here showed that 2.5 mg caffeine $ml^{-1}$ completely inhibited the excision of pyrimidine dimers in wild-type B.fragilis cells under anaerobic conditions, and that reversal of this effect could not be achieved by a further 1 h holding in the absence of caffeine. This result agrees with the observations of Campbell and Yasbin (1984) on the effect of caffeine on dimer excision in N.gonorrhoeae. Furthermore, irradiated B.fragilis cells held in

2.5 mg caffeine $ml^{-1}$ showed a 2-3 fold decrease in viability when plated on caffeine-free medium as compared to unirradiated cells held and plated under the same conditions. These results indicate that in B.fragilis caffeine appears to act specifically at the level of dimer excision and not through the inhibition of viability of B.fragilis cells in general. The site of action of caffeine in the inhibition of dimer removal is further examined in Chapter IV.

The semi-quantitative measurement of dimer removal from densitometer tracings of photographic negatives by means of peak triangulation was sufficiently accurate to express the major differences and similarities between the B.fragilis wild-type and mutant strains. Extension of the work to accurate quantitation of the numbers of dimers involved would, however, require methods which take into account the logarithmic nature of the photographic process. The methods of Pulleyblank et al. (1977) and Prunell (1980) corrected the densitometric data for the film response function but still retained possible errors due to the rectangular shape of the probing beam and the non-uniform distribution of DNA across the lanes of a gel.

The most accurate method to date has been developed by Freeman et al. (1986). In this method, each lane of DNA in the photographic negative is scanned and analysed by a computer system. Analysis programmes convert film darkening to DNA concentration and, by comparison with the

mobility of standards of known molecular length, calculate the number average molecular length of the population. From the molecular lengths of enzyme treated and untreated irradiated DNAs, the frequency of single strand breaks and hence the number of dimers present in the treated DNA may be calculated. The accuracy of this method compares favourably with the results obtained using alkaline sucrose gradient centrifugation of labelled DNA.

The alkaline agarose gel electrophoresis method used in this work clearly indicates that there is efficient dimer removal (as reflected by the loss of enzyme-sensistive sites) from the DNA of UV-irradiated B.fragilis during holding under aerobic and anaerobic conditions. The method does not, however, distinguish between excision repair mediated by DNA glycosylase/endonuclease activity or by endonucleases alone (Chapter I). The most definitive assay to distinguish DNA glycosylase activity from endonuclease activity is the direct demonstration of the release of free base by the former by means of chromatographic analysis of radiolabelled DNA (Duncan, 1981). This method would not, however, detect sequential glycosylase/endonuclease activity where the dimer could eventually be excised as part of an oligonucleotide (Fig. 3.1). In B.fragilis where high levels of radiolabelling are not easily achieved, an alternative route would be the cloning of the gene(s) involved and in vitro analysis of their mode of action (Chapter V).

# CHAPTER IV

# ALKALINE SUCROSE GRADIENT SEDIMENTATION STUDIES OF DAMAGED B.FRAGILIS DNA.

## SUMMARY

Alkaline sucrose gradient sedimentation techniques were used to investigate the direct effects of certain DNA damaging agents on B.fragilis DNA as well as the enzymatic responses induced during repair of this damage.

The direct strand-breakage effect of far-UV irradiation on B.fragilis DNA was very small, but irradiation induced metabolically regulated strand-breakage and resynthesis in the wild-type strain under both aerobic and anaerobic conditions. Only 50% of the strand-breaks accumulated under anaerobic conditions were removed during buffer-holding, but under replicating conditions the original high $M_r$ of the DNA was regained. The UVS9 and MTC25 mutant strains showed levels of strand-breakage comparable to the relative dimer removal efficiencies of these strains as assayed by the M.luteus pyrimidine dimer endonuclease enzyme probe.

Under aerobic conditions, oxygen exposure induced low levels of DNA strand-breaks which were enhanced by prolonged exposure of the DNA under alkaline conditions. DNA strand-breakage and complete resynthesis were observed

in the UV-irradiated wild-type strain and to a markedly reduced level in the UVS9 and MTC25 mutant strains.

Caffeine exerted a direct strand-breaking effect on wild-type DNA under anaerobic conditions and did not inhibit either the further breakage of the DNA or its resynthesis following UV irradiation.

Mitomycin C was metabolically activated under anaerobic conditions and formed DNA cross-links in B.fragilis DNA. At lethal doses, it also caused major DNA degradation and loss of TCA precipitable material from the DNA on the gradients.

## 4.1 INTRODUCTION

Sedimentation of radiolabelled DNA in alkaline sucrose gradients (McGrath and Williams, 1966) is a powerful technique for the measurement of single-strand breaks and cross-links in DNA. It is a technique capable of high precision and requires only a few nanocuries of DNA for each sample as opposed to chromatographic techniques which need microcurie amounts (Hays et al., 1985).

In the context of the present study, an analysis of the conditions under which single-strand breaks and DNA cross-links could occur is of interest from two points of view. It is a way of observing some of the direct effects of certain chemical and physical damaging agents on the DNA, as well as monitoring the enzymatic repair activity induced in response to this damage.

The aspects which are covered in this chapter are outlined below.

### 4.1.1 The effect of oxygen and its radicals on the DNA of an anaerobic organism.

The effect of oxygen and its radicals on macromolecular synthesis in B.fragilis, and on the induction of novel repair systems in this organism has been reviewed in Chapter I. A question arising from this work is whether oxygen (or its radicals) is able to act directly on the DNA as a damaging agent by generating nicks.

Moody and Hassan (1982) showed that the superoxide radical ($O_2^-$), formed by the reduction of oxygen from its ground state, is highly reactive and can damage DNA by causing single-strand breaks. This is done by the superoxide radical directly or by the secondary production of the peroxyanion. Hydrogen peroxide ($H_2O_2$) has been found to increase the number of single-strand breaks in E.coli (Hartman and Eisenstark, 1978; Hagensee and Moses, 1986), S.typhimurium (Yoakum and Eisenstark, 1972), and T7 phage (Ananthaswamy and Eisenstark, 1976). The nicking effect of oxygen and oxygen radicals on the DNA of an anaerobic organism is therefore of interest.

4.1.2 UV irradiation

Cyclobutane-type pyrimidine dimers are the major photoproduct in DNA irradiated with (254 nm) far-UV light (Chapter I). Breakage of the polynucleotide chain has, however, also been observed (Smith and Hanawalt, 1969; Setlow, 1967), and DNA strand-breaks due to hydrolysis of alkali-labile photoproducts (pyrimidine-pyrimidine 6,4 adducts) could occur (Haseltine, 1983).

Strand-breakage by biologically significant levels of far-UV light should therefore be examined in the anaerobic situation.

4.1.3 Mitomycin C treatment

The molecular mechanism of mitomycin C action was first established by Iyer and Szybalski (1963) who showed that exposure of B.subtilis and E.coli cells to inhibitory concentrations of mitomycin C resulted in covalent cross-linking of complementary DNA strands. Recently, the bifunctional alkylation action of mitomycin C has been proved directly by Tomasz et al. (1987) through isolation and chemical characterization of the adduct involved. Cross-linkage is generally recognized as the main cytotoxic effect of this antibiotic, probably due to inhibition of DNA synthesis (Kersten, 1971). However, Lown (1983) has reported that oxidation of the hydroquinone moiety of the DNA-bound antibiotic generates the semi-quinone, $O_2^-$, $H_2O_2$, and $OH^-$ which can cause single-strand breaks in DNA.

Partial breakdown of DNA accompanied by resynthesis is thought to reflect the excision phenomenon connected with mitomycin C alkylated DNA repair (Boyce and Howard-Flanders, 1964). Extensive breakdown of DNA has been reported in certain organisms in the presence of $Mg^{2+}$ ions. This process is temperature sensitive and leads to the formation of mononucleotides and free bases (Reich et al., 1961; Shiio et al., 1962).

Alkaline sucrose gradient sedimentation has been used to observe the formation and enzymatic removal of

DNA cross-links induced by psoralen-plus-light in E.coli (Cole et al., 1976), and Pera et al. (1981) found it to be a sensitive technique (comparable to alkaline elution studies) for the quantitation of the formation and loss of Cisplatin-induced cross-links in Chinese hamster cells. It is proposed that the same technique could be used to examine the molecular effect of mitomycin C on B.fragilis DNA.

4.1.4 Enzymatic response of repair endonuclease(s), glycosylase(s) or both to damaged DNA.

The mechanisms by which E.coli and several other prokariotic and eukariotic organisms achieve the reversal, the removal and/or the tolerance of UV-induced DNA damage, as well as the enzymatic events which have been found to lead to the excision of pyrimidine dimers in bacterial and mammalian systems have been reviewed in Chapter I. It is clear, therefore, that whether excision repair is initiated by an endonuclease, or a glycosylase followed by an endonuclease, the effect at the DNA level is the production of nicks or strand-breaks. Failure to generate such nicks has been shown to inhibit dimer excision and be the cause of UV-sensitivity in certain mutant strains (Chapter I).

Excision repair after UV-irradiation can, therefore, be studied by following the number of breaks appearing in pre-labelled DNA during post-irradiation incubation, as

well as the disappearance of these breaks as a result of resynthesis and ligation of the repaired DNA. This approach has been used to monitor the DNA incision capabilities of several M.radiodurans mutant strains (Tempest and Moseley, 1980; Evans and Moseley, 1983). The method of alkaline sucrose gradient sedimentation has also been used to observe post-replication repair in excisionless mutant strains such as E.coli uvrA mutants (Howard-Flanders and Rupp, 1981) and B.subtilis uvr$^-$ mutants (Dodson and Hadden, 1980).

The physiologically observed joint-sensitivity of mutants of certain organisms, but not others, to both mitomycin C and UV-irradiation has been discussed in Chapter 2.1. Kohn (1983) has reviewed possible models for the excision of mitomycin C induced cross-links. These involve similar enzymatic events to those occurring in the removal of pyrimidine dimers. In organisms where joint-sensitivity is observed, it is postulated that there is joint recognition of bulky damage generated either by UV or mitomycin C by the UV repair enzymes (Rupp et al., 1982). In M.radiodurans, however, it is proposed that two separate UV endonucleases are present, one of which recognizes only pyrimidine dimers, and the other both pyrimidine dimers and mitomycin C cross-links as well as other alkylation damage (Evans and Mosely, 1983).

Comparable studies of the DNA damage and repair mechanisms of B.fragilis are still at an early stage. The results reported in Chapter III (Abratt et al., 1986) showed that in wild-type B.fragilis cells, pyrimidine dimers (UV dimer endonuclease sensitive sites) were generated during UV irradiation, and that removal of pyrimidine dimers was efficiently achieved during anaerobic holding in buffer. The B.fragilis mitomycin C-sensitive mutant (MTC25) showed slightly reduced and the UV-sensitive mutant (UVS9) markedly reduced levels of dimer excision compared to the wild-type organism. Under aerobic conditions both the wild-type and MTC25 mutant showed equally good levels of dimer removal, while it remained much lower in the UVS9 strain.

The B.fragilis wild-type and mutant strains available for this study (Abratt et al., 1985) have, therefore, the potential for elucidating aspects of the mechanism of dimer removal in this organism.

## 4.2 METHODS

### 4.2.1 BACTERIA

The *B.fragilis* wild-type strain (Bf-2), and mitomycin C-sensitive (MTC25) and UV-sensitive (UVS9) mutants of this strain, were used ; they have been described in 2.2.1. In addition *E.coli* K12 strains AB1157 ($F^-$ wild-type), X2819 (*thy*$^-$, *recA*$^-$), CSR603 (*recA*$^-$ *uvrA*$^-$), and AB1886 (*uvrA*$^-$), were used (Table 4.1).

### 4.2.2 MEDIA

For the *B.fragilis* strains, Difco BHI broth and agar, one-quarter-strength Ringer's solution dilution buffer, and stringent anaerobic conditions where necessary were used as described in 2.2.2, 2.2.3.

The *E.coli* strains were maintained routinely on Luria agar at 37°C, and Luria broth cultures were grown at 37°C with vigorous aeration for 12 h. The Luria broth and agar were supplemented with 100 $\mu$g $ml^{-1}$ thymine for the *thy*$^-$ strain. *E.coli* irradiation, dilution and holding were done in phosphate buffer (Appendix I).

### 4.2.3 DNA LABELLING

*B.fragilis* strains were sub-cultured at least 3 times at 48 h intervals on BHI agar and then inoculated into BHI broth for overnight incubation at 37°C. Pre-warmed minimal medium (3 ml in sterilin tubes) was inoculated with 0.1 ml of the overnight culture, and incubated for

TABLE 4.1

*E. coli* K-12 strains used in this study.

| Strain | Genotype | Origin |
|---|---|---|
| AB1157 | $F^-$ wild-type | [1] CGSC1157 |
| AB1886 | *uvrA6* | CGSC1886 $F^-$ |
| CSR603 | $F^-$ *thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 gyrA98(nalA98) rpsL31 tsx-33* $\lambda^-$ *supE44* | CGSC |
| X2819 | $F^-$ *lacY1 glnV44 galK2 galT22 recA56* $\Delta$*thyA57 metB1 hsdR2* $\lambda$ *cI857 b2 red3 S7* | [2] Roy Curtiss III |

[1] CGSC - *E. coli* Genetic Stock Centre, Yale University
[2] Department of Biology, Washington University

approximately 2.5 h at 37°C to early log phase ($A_{600}$ 0.15). Pre-reduced deoxyadenosine (final concentration 50 μg $ml^{-1}$) and $^{3}H$-thymidine (Amersham, specific activity 70-90 Ci $mmol^{-1}$; final concentration 10 μCi $ml^{-1}$) were added and incubation continued for approximately 1.5 - 2.0 h to $A_{600}$ 0.3. Under anaerobic conditions, 1 ml culture was removed to an Ependorf tube, the cells harvested by centrifugation and then washed once in RS. The pellet was resuspended in 1 ml RS (aerobic or anaerobic), and kept under either aerobic or anaerobic conditions while the level of incorporation of the label (in 10 μl) was measured on a Packard scintillation counter.

E.coli labelling was done during overnight growth of 2 ml volumes of the strains (4.2.2) in the presence of 50 μg $ml^{-1}$ deoxyadenosine and 10 μCi $ml^{-1}$ $^{3}H$-thymidine (final concentrations). The culture was harvested by centrifugation, washed once in E.coli holding buffer, and resuspended in buffer at a concentration of about $10^{8}$ cells $ml^{-1}$ before measuring for incorporation of label.

Labelled bacteriophage lambda DNA, for use as a standard in calibrating the alkaline sucrose gradients, was prepared by end-labelling the cos ends of the linear molecules with ($^{32}P$) dCTP (Amersham, specific activity 3000 Ci $mmol^{-1}$) according to the method of Maniatis et al. (1982).

4.2.4 UV IRRADIATION AND HOLDING

Methods for irradiating cells under aerobic or anaerobic conditions were as described in 2.2.3. Labelled B.fragilis cells were diluted 10-fold into a final volume of 5 ml RS in a glass Petri dish on ice (approximately $10^7$ cells $ml^{-1}$) and irradiated with a dose of 15 J $m^{-2}$ $s^{-1}$. The cells were then either loaded directly on to the gradient or held at 30°C for various time intervals before loading.

In experiments to show the effect of caffeine on the nicking of DNA, the appropriate concentration of caffeine (0.1- 2.5 mg $ml^{-1}$ as indicated) was added to the holding buffer of either irradiated or unirradiated wild-type cells under anaerobic conditions.

Labelled E.coli cells were diluted $10^{-1}$ into phosphate buffer on ice (approximately $10^7$ cells $ml^{-1}$) and irradiated with a UV dose of 10 J $m^{-2}$ (repair deficient strains) or 20 J $m^{-2}$ (wild-type) (Howard-Flanders and Rupp, 1981) before loading immediately onto the gradients or holding at room temperature.

Irradiation and holding were carried out under very subdued light conditions and holding times were terminated by placing the samples on ice before loading on to the gradients.

4.2.5 POST-IRRADIATION NICKING OF PRE-LABELLED DNA UNDER REPLICATING CONDITIONS

$^{3}$H-labelled wild-type B.fragilis cells (4.2.3.) were resuspended in 5 ml fresh, pre-warmed MM broth and incubated at 37°C for 1 h to ensure the resumption of active replication (Schumann et al, 1983). The cells were then irradiated in this broth (25 J $m^{-2}$ $s^{-1}$; 0.1% survival) as described in 4.2.4. Samples (0.2 ml) were removed either immediately after irradiation or after holding in the MM at 37°C for various lengths of time, harvested anaerobically by centrifugation, and resuspended in 1 ml anaerobic RS before loading on to the gradients.

4.2.6 EFFECT OF MITOMYCIN C ON DNA

Labelled B.fragilis cells were treated either with 1.0 µg $ml^{-1}$ or 0.5 µg $ml^{-1}$ of mitomycin C as indicated, under anaerobic conditions for various lengths of time, either at 4°C (on ice) or 37°C. The reaction was terminated by placing the samples on ice for 5 min before centrifuging and resuspending the cells in cold anaerobic RS. The cultures were diluted $10^{-1}$ in RS before loading on to the gradients.

4.2.7 ALKALINE SUCROSE GRADIENT SEDIMENTATION

Alkaline sucrose gradient analysis was performed as described by Peak and Peak (1982), taking into account the precautions against the formation of artefacts cited by Lett (1981). Sets of 6 linear 5-20% alkaline sucrose

gradients (final volume of each = 4.8 ml) were prepared simultaneously using a single gradient mixer connected to a manifold and a six-channel peristaltic pump. This ensured standardization of the gradients used within a particular experiment. The radiolabelled cells (0.1 ml) were layered on to the top of the gradient and lysed in situ by the addition of an equal volume of alkaline lysis buffer containing 0.1% SDS. The gradients contained, on an average, between $5 \times 10^3$ and $1 \times 10^4$ cpm, and the number of cells loaded on to each gradient was between $1\text{-}5 \times 10^6$.

Lysis was for at least 30 min and not more than 5 h at $4^{\circ}C$, after which the gradients were centrifuged at 35,000 rpm in a Beckman SW 50.1 rotor at $18^{\circ}C$ for 65 min. The gradients were harvested immediately by simultaneous pumping from the bottom of the tubes using the six-channel peristaltic pump in the reverse direction, and the fractions (30 fractions per gradient) collected on graduated filter paper strips supported on a non-wetting surface and marked with a soft lead pencil. The DNA was precipitated by washing the strips in 5% trichloroacetic acid for 10 min, followed by 2 x 10 min washes in 95% ethanol. The strips were air dried, cut up into fraction lengths and counted in 4 ml scintillation fluid using a Packard scintillation counter.

## 4.2.8 MOLECULAR WEIGHT CALCULATIONS

The number average molecular weights ($M_n$) and the number of single-strand breaks (SSB's) caused by the various treatments of the DNA were calculated according to the methods of Peak and Peak (1982), and Youngs and Smith (1976) - see appendix II for details.

Single-strand molecular weights were proportional to the 2.62 power of the distance sedimented as compared with E.coli phage T4 DNA ($55 \times 10^6$ dalton). The number average molecular weights ($M_n$) were calculated as described by Ley (1973) using computer analysis from the formula:

$$M_n = \frac{\leq R_i}{\leq (R_i/M_i)}$$

where $R_i$ is the percentage radioactivity in the ith fraction and $M_i$ is the molecular weight of the DNA in the ith fraction.

The number of SSB's was calculated as reported by Tyrrell et al. (1974) and Peak and Peak (1982) where

$$SSB = \frac{M_n(\text{initial})}{M_n(\text{final})} - 1 \text{ per molecule} = (A)$$

and

$$(A) \; / \; M_n(\text{initial}) \times 10^2 = \text{number SSB's per } 10^8 \text{ daltons}$$

## 4.3 RESULTS

The term "strand-break" is used as opposed to "nick" since the method used does not enable a distinction to be made between single- and double-stranded DNA breaks, as well as alkali-labile sites in the DNA which may be converted into breaks under alkaline gradient conditions. The results, therefore, reflect the total number of breaks generated by these three mechanisms (4.4).

In experiments to determine the kinetics of strand-breakage and repair, the term "maximum number of accumulated breaks" is used to describe the highest number of breaks observed in the DNA at a particular time. It does not refer to the total number of breaks generated in the course of repair (4.4).

The results reported here, while showing definite trends, are semi-quantitative since they were influenced by the variable nature of a large plasmid co-sedimenting with the chromosomal DNA (Fig. 4.4A). The behaviour and significance of this plasmid are discussed further in 4.4.

The gradient profiles presented here were confirmed in 3-5 repeat experiments performed in each case. The results shown in Tables 4.2 and 4.3 reflect the average numbers of breaks calculated from the gradient data with variations from the mean as indicated.

### 4.3.1 STRAND-BREAKAGE OF UV-IRRADIATED DNA DURING ANAEROBIC AND AEROBIC HOLDING IN BUFFER

In all the experiments involving irradiation of cells, the UV dose administered had a very low capacity for inducing direct DNA strand-breakage. The average number of strand-breaks and alkali-labile sites induced under aerobic and anaerobic conditions at $4^0$C were approximately $5 \times 10^{-3}$ and $2 \times 10^{-3}$ per $10^8$ daltons per J $m^{-2}$ respectively. This result is in agreement with the breakage efficiency obtained by Tyrrell *et al.* (1974) under similar conditions.

Calibration of the gradient system using known *E.coli* mutants gave results comparable to the behaviour reported in the literature. *E.coli* K12 wild-type strain AB1157 rapidly introduced incision breaks in the DNA as part of dimer excision (Fig. 4.1A). *E.coli* K12 strain AB1886 (*uvrA*), however, showed no nicking of the irradiated DNA as a result of the inactive *uvrA* gene (Howard-Flanders and Rupp, 1981) (Fig. 4.1B). *E.coli* K12 strain X2819 (*recA thy*) showed strand-breakage and resynthesis under the liquid holding recovery repair conditions of Harm (1966) (Fig. 4.2A,B). Excision repair in *E.coli* was completed in approximately 60-90 min (Youngs and Smith, 1973; Youngs *et al.*, 1974). *E.coli* K12 strain CSR603 (*recA*, *uvrA*) showed progressive degradation of the irradiated DNA up to 90 min post-irradiation holding with no indication of

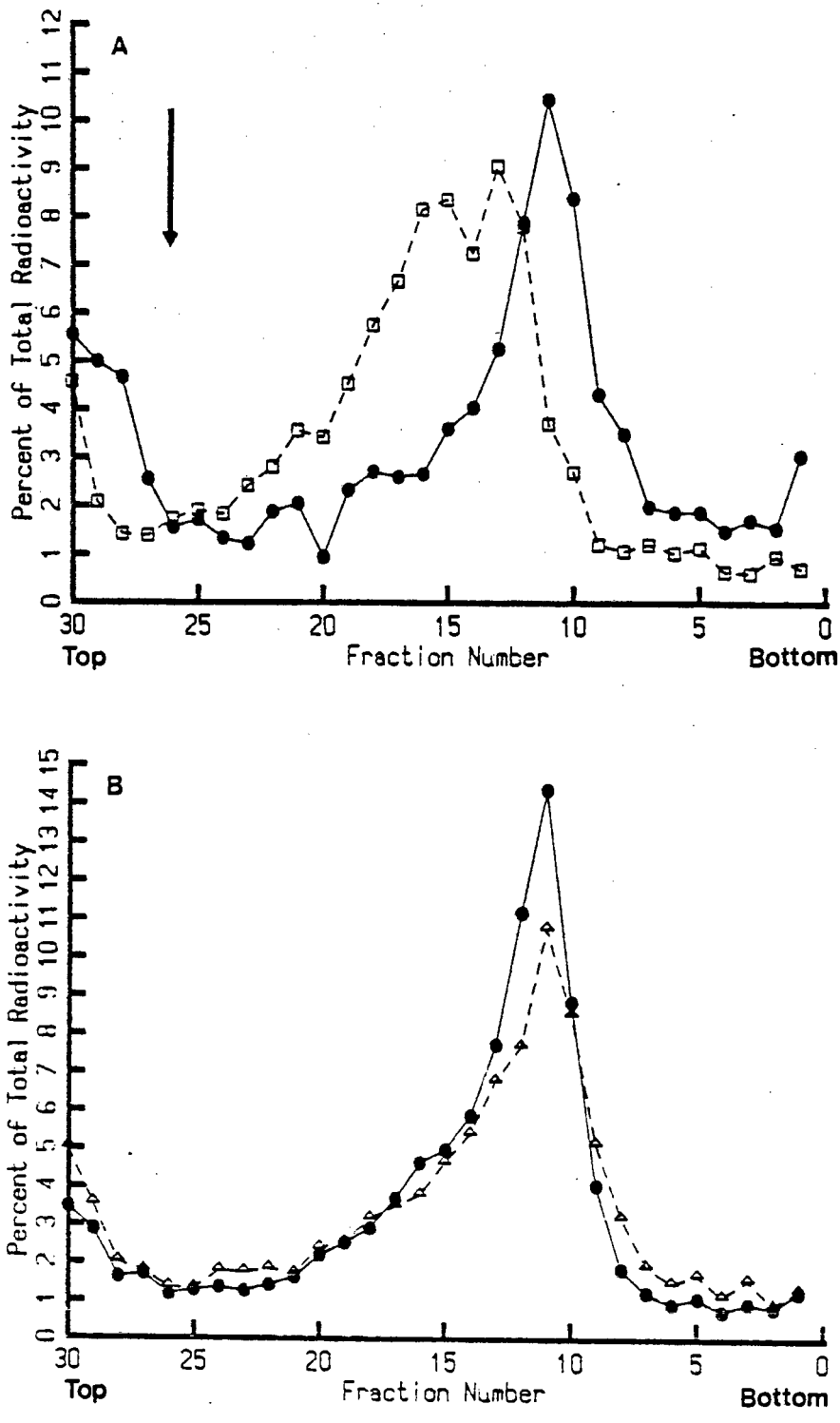



FIGURE 4.1: Alkaline sucrose sedimentation analysis of UV-irradiated E.coli AB1157, wild-type, (20 J $m^{-2}$) and excisionless mutant AB1886 (10 J $m^{-2}$) strains.
Gradients are shown at the post-irradiation holding time giving the maximum change in $M_n$ of the DNA.
A, AB1157; ●—● control; □- - -□ UV, held for 5 min.
B, AB1886; ●—● control; △- - -△ UV, held for 15 min.
Arrow, sedimentation of λDNA.

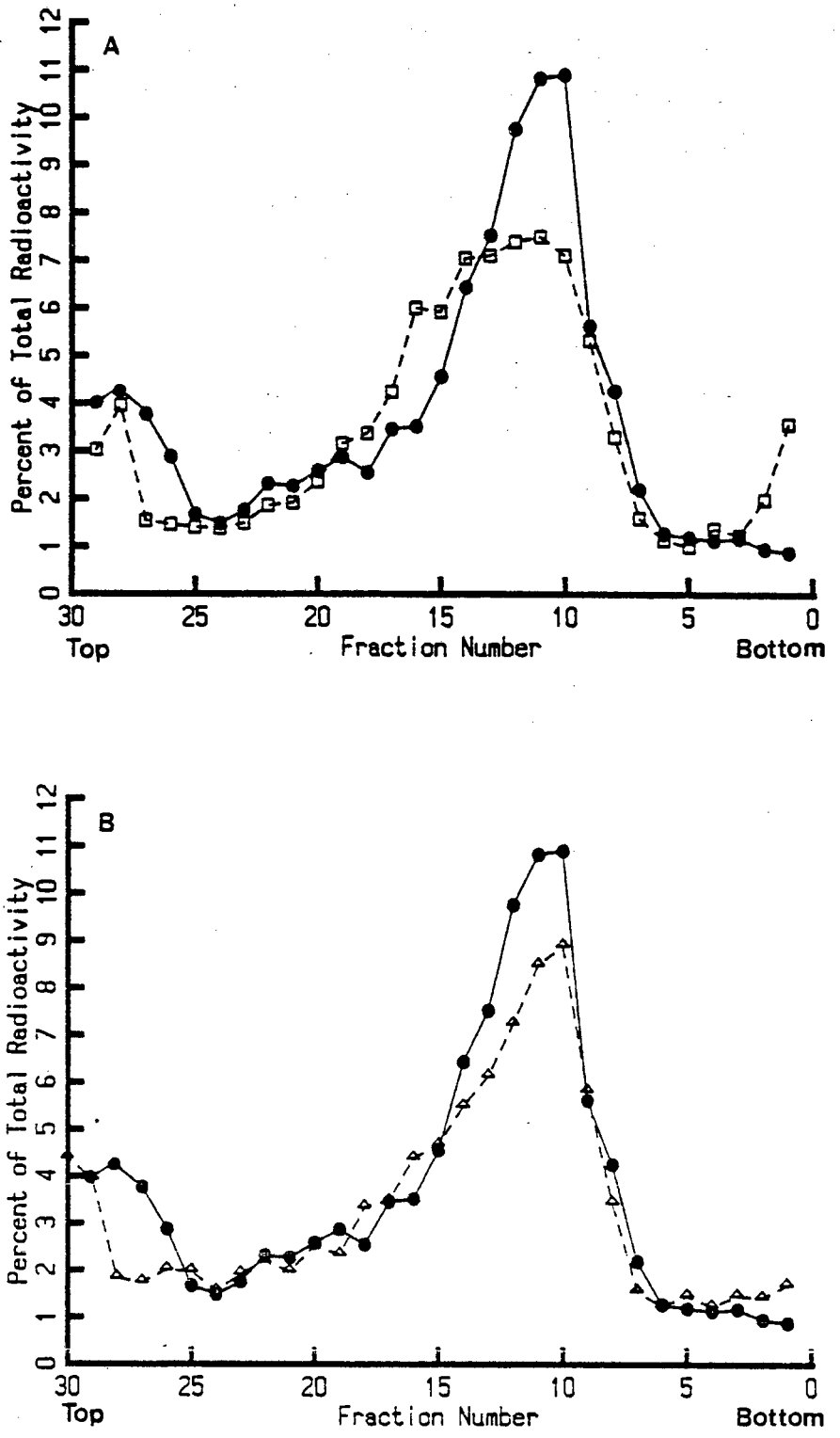



FIGURE 4.2: Alkaline sucrose sedimentation analysis of irradiated (10 J $m^{-2}$) E.coli K12 X2819 (recA).

A, ●——● control, □- - -□ irradiated, held for 15 min.

B, ●——● control, △- - -△ irradiated, held for 90 min.

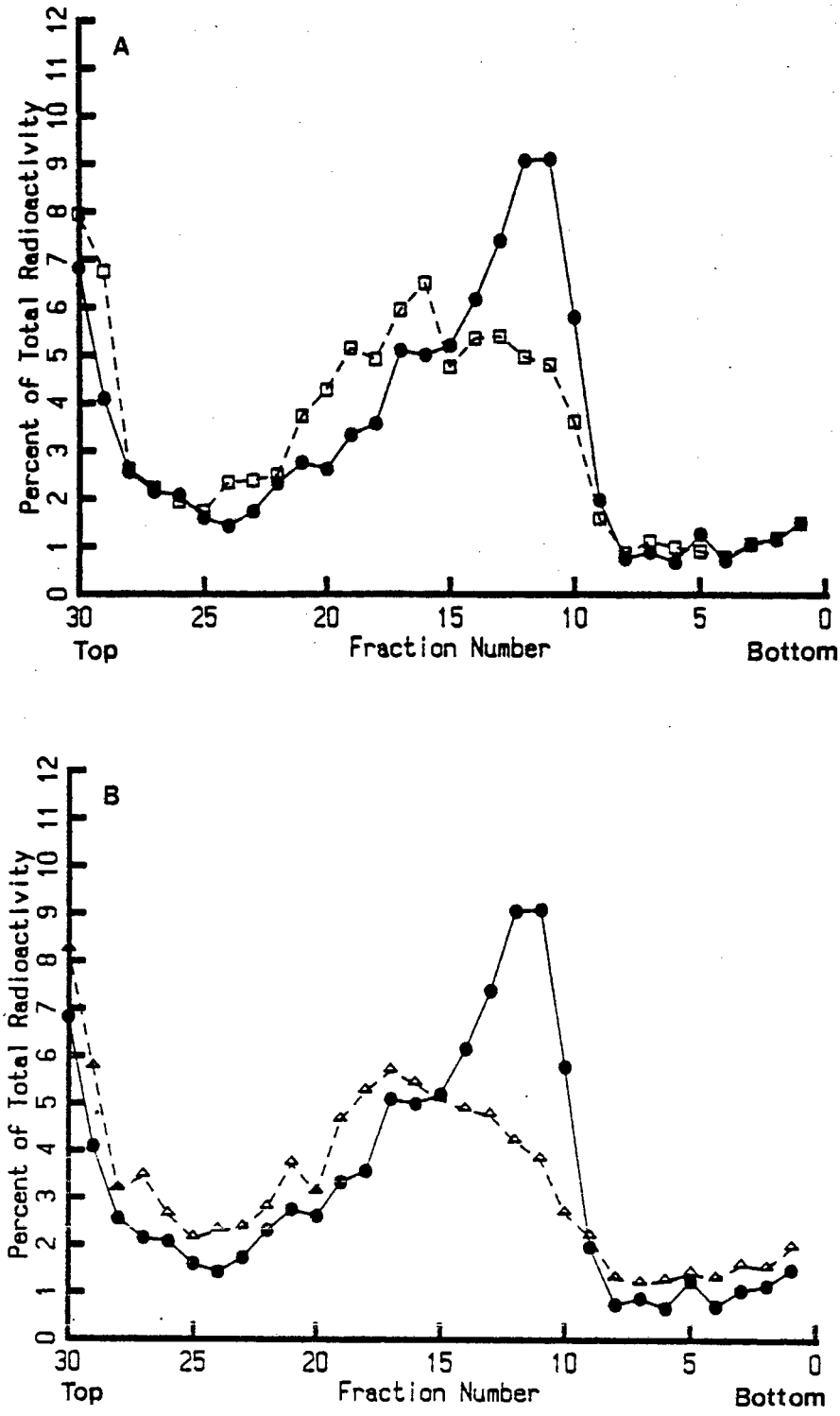



FIGURE 4.3: Alkaline sucrose sedimentation analysis of irradiated (10 J $m^{-2}$) E.coli K12 CSR603 (recA,uvrA).

A, ●——● control; □- -□ irradiated, held for 15 min.

B, ●——● control; △- -△ irradiated, held for 90 min.

repair resynthesis (Fig. 4.3A,B). This behaviour is typical of the so-called recA deficient "reckless" mutants where extensive degradation of DNA occurs following UV irradiation (Clark, 1973).

The average number molecular weights ($M_n$) of the DNA of the various E.coli strains were between 170 and 120 x $10^6$ daltons depending on the strain used, which is in agreement with the range of values (150 and 110 x $10^6$ daltons reported by Youngs and Smith (1976) and Tyrrell et al. (1974) respectively.

Under anaerobic holding conditions, the irradiated wild-type B.fragilis strain generated DNA strand-breaks which reached an accumulation maximum at 40 min after irradiation of 0.9 - 1.0 strand-breaks per $10^8$ daltons (Fig. 4.4A). After this time the number of breaks was reduced to approximately 0.5 per $10^8$ daltons at 60 min holding, and the $M_n$ of intact DNA was not regained even after 2 h holding (Fig. 4.4B; Table 4.2A).

Under the same conditions, the UVS9 (UV/mitomycin C -sensitive) and the MTC25 (mitomycin C- sensitive) strains showed reduced levels of strand-breakage compared to the wild-type. Maximum accumulated breakage in the UVS9 strain was 0.3 - 0.4 per $10^8$ daltons occurring at 20 min post-irradiation holding (Fig. 4.5A; Table 4.2A) and this was reduced to approximately 0.15 -0.2 breaks during 1-2 h

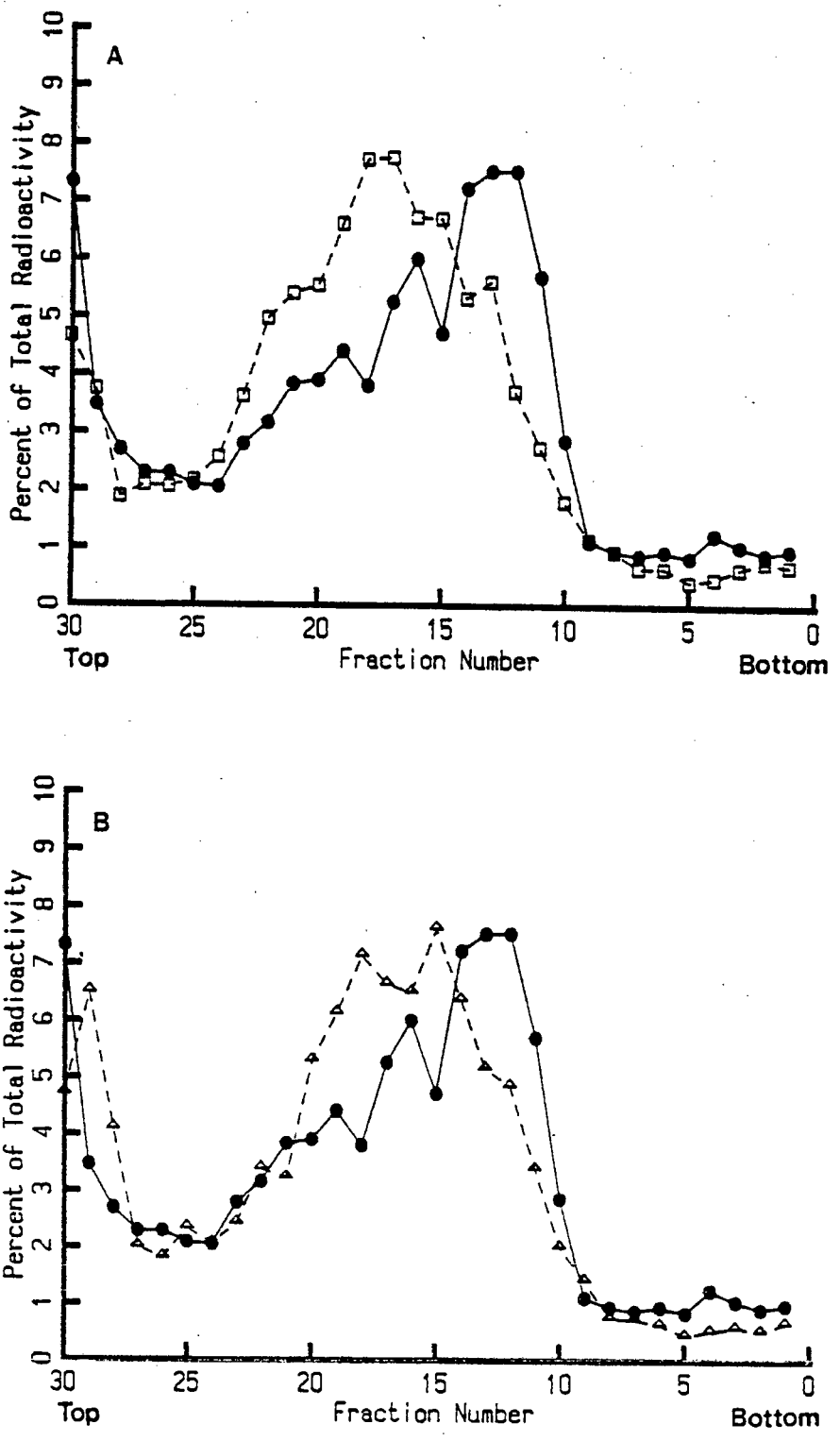



FIGURE 4.4: Alkaline sucrose gradient sedimentation of B.fragilis wild-type, irradiated (15 J $m^{-2}$) and held in anaerobic buffer.

A, ●——● control; □- - -□ irradiated, held for 40 min.

B, ●——● control; △- - -△ irradiated, held for 60 min.

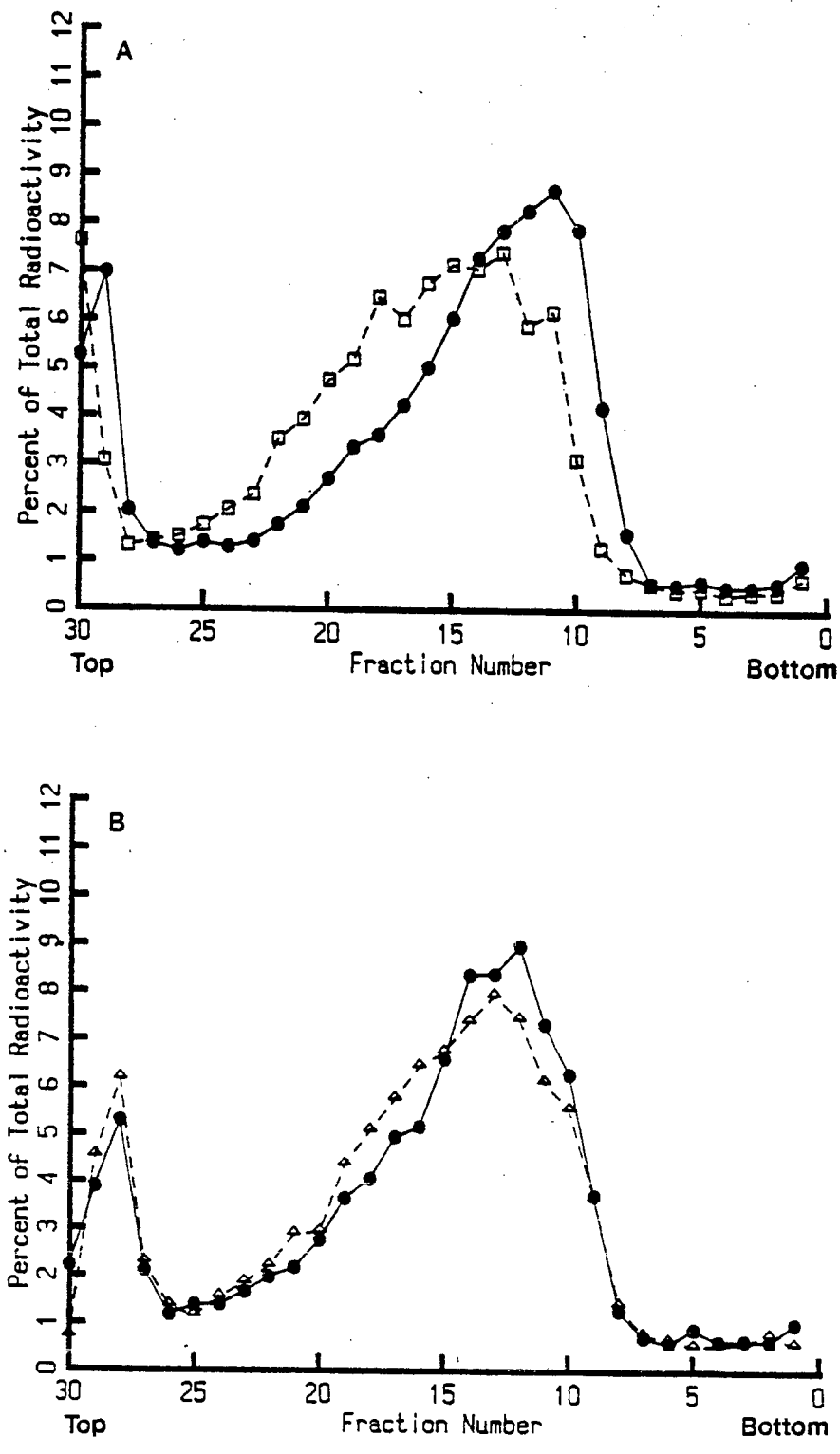



FIGURE 4.5: Alkaline sucrose gradient sedimentation of *B.fragilis* UVS9, irradiated (15 J $m^{-2}$) and held in anaerobic buffer.

A, ●——● control; □- - -□ irradiated, held for 20 min.

B, ●——● control; △- - -△ irradiated, held for 60 min.

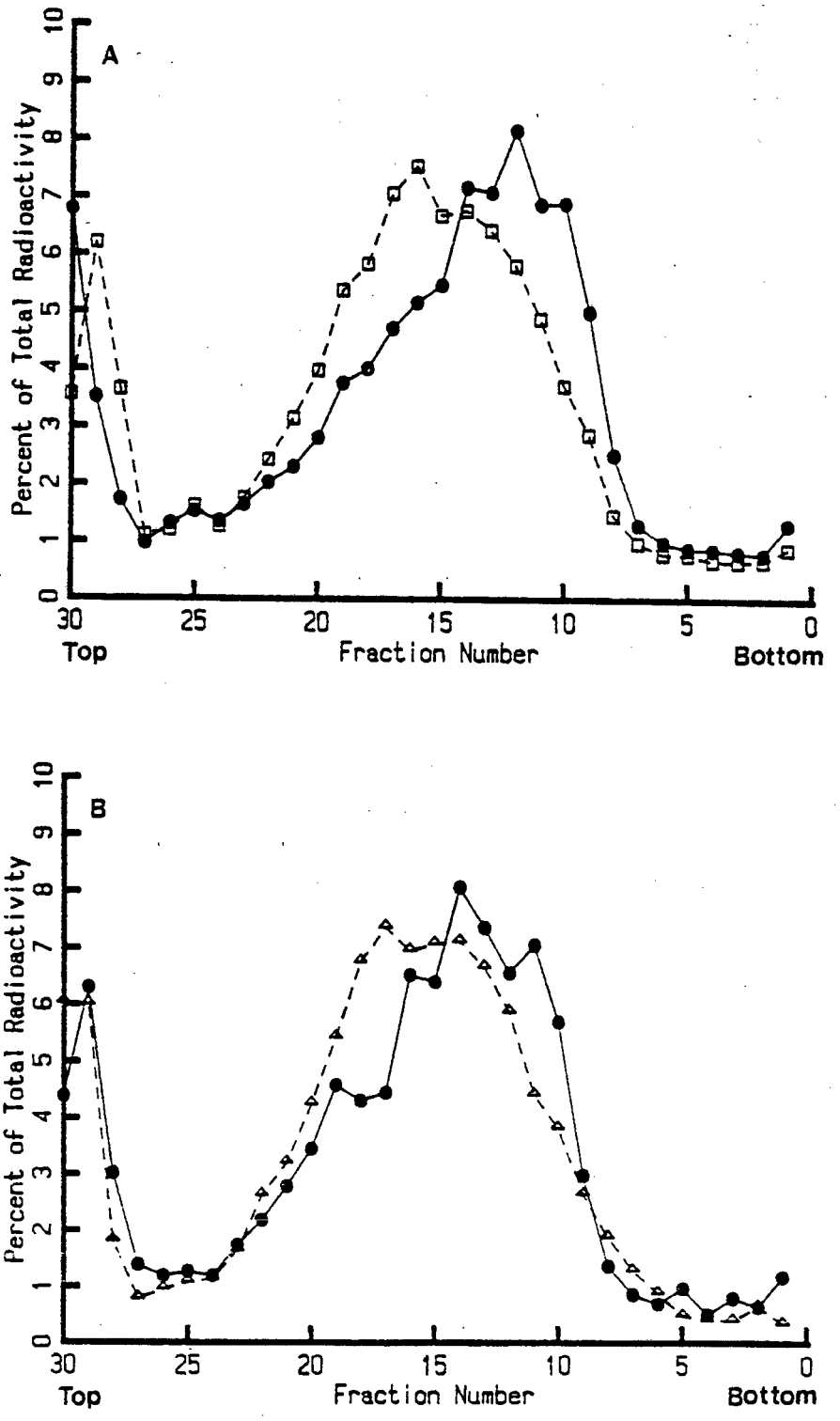



FIGURE 4.6: Alkaline sucrose gradient sedimentation of B.fragilis MTC25, irradiated (15 J $m^{-2}$) and held in anaerobic buffer.

A, ●——● control; □- - -□ irradiated, held for 20 min.

B, ●——● control; △- - -△ irradiated, held for 60 min.

holding (Fig. 4.5B). The MTC25 strain was intermediate in breakage capacity between the wild-type and the UVS9 strains (Fig. 4.6B; Table 4.2A) reaching a maximum of 0.4 - 0.5 breaks per $10^8$ daltons at 20 - 40 min after irradiation, and a minimum of approximately 0.2 - 0.35 over the 1-2 h holding period (Fig. 4.6B).

Under aerobic conditions, the $M_n$ of the unirradiated DNA from the wild-type strain was slightly lower than that of the DNA from cells which had been washed and resuspended in anaerobic RS, being 106 and 136 x $10^6$ daltons respectively. Holding of wild-type cells aerobically generated an accumulated maximum number of approximately 0.5 strand-breaks per $10^8$ daltons after 5 - 10 min, and the original aerobic $M_n$ was regained after 60-90 min holding (Figs 4.7A,B; Table 4.2B).

The UVS9 showed lower levels of strand-breakage (maximum 0.15 - 0.25 breaks per $10^8$ daltons at 5 min) and the gaps were rapidly resealed after 15 min holding (Figs 4.8A,B; Table 4.2B).

The MTC25 mutant strain showed very low levels of strand-breakage which did not excede 0.25 per $10^8$ daltons in six separate repeat experiments over the range of holding times indicated (Figs 4.9A,B; Table 4.2B).

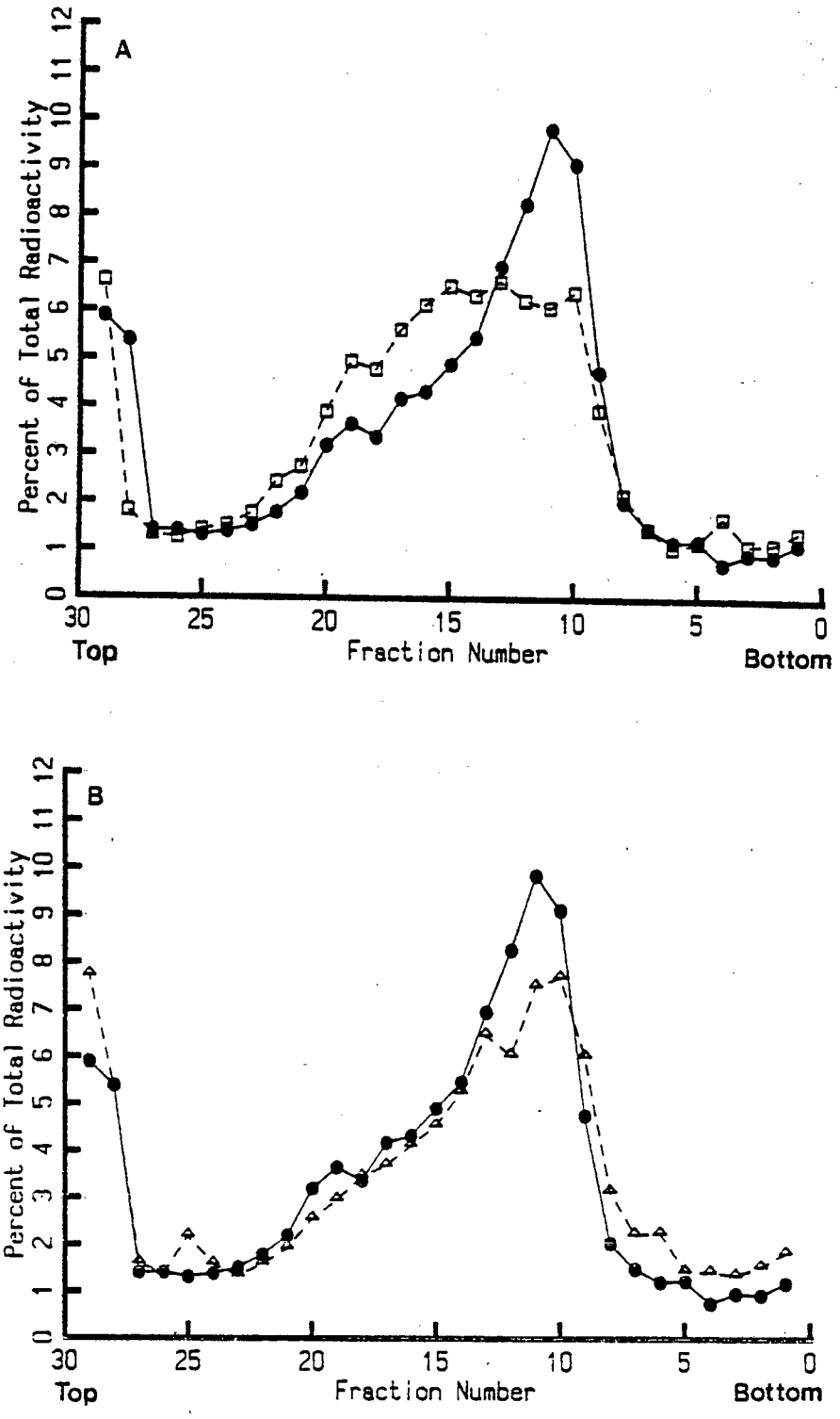



FIGURE 4.7: Alkaline sucrose gradient sedimentation of B.fragilis wild-type irradiated (15 J $m^{-2}$) and held in aerobic buffer.

A, ●——● control; □- - -□ irradiated, held for 10 min.

B, ●——● control; △- - -△ irradiated, held for 60 min.

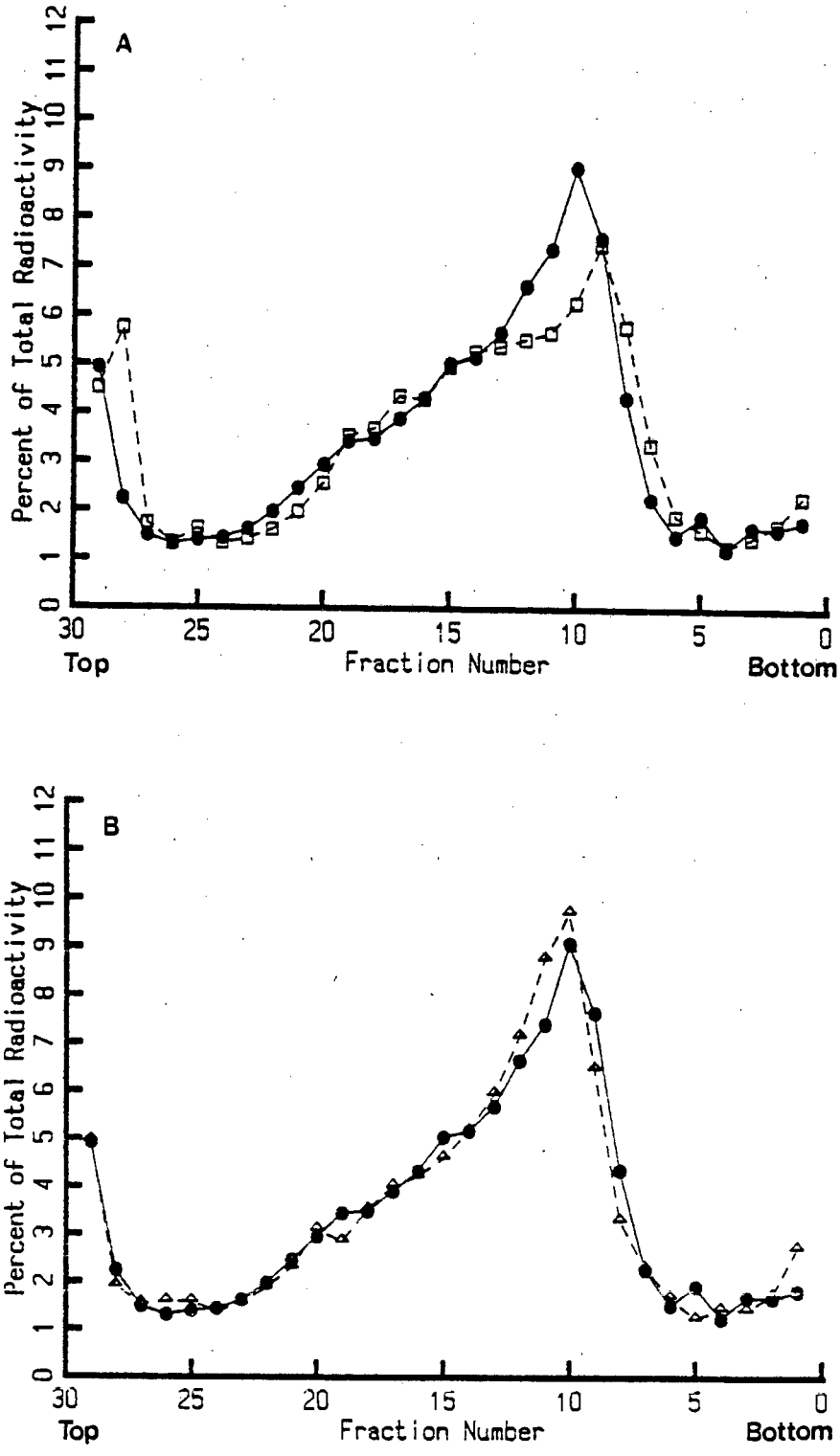



FIGURE 4.8: Alkaline sucrose gradient sedimentation of B.fragilis UVS9 irradiated (15 J $m^{-2}$) and held in aerobic buffer.

A, ●——● control; □- - -□ irradiated, held for 5 min.

B, ●——● control; △- - -△ irradiated, held for 15 min.

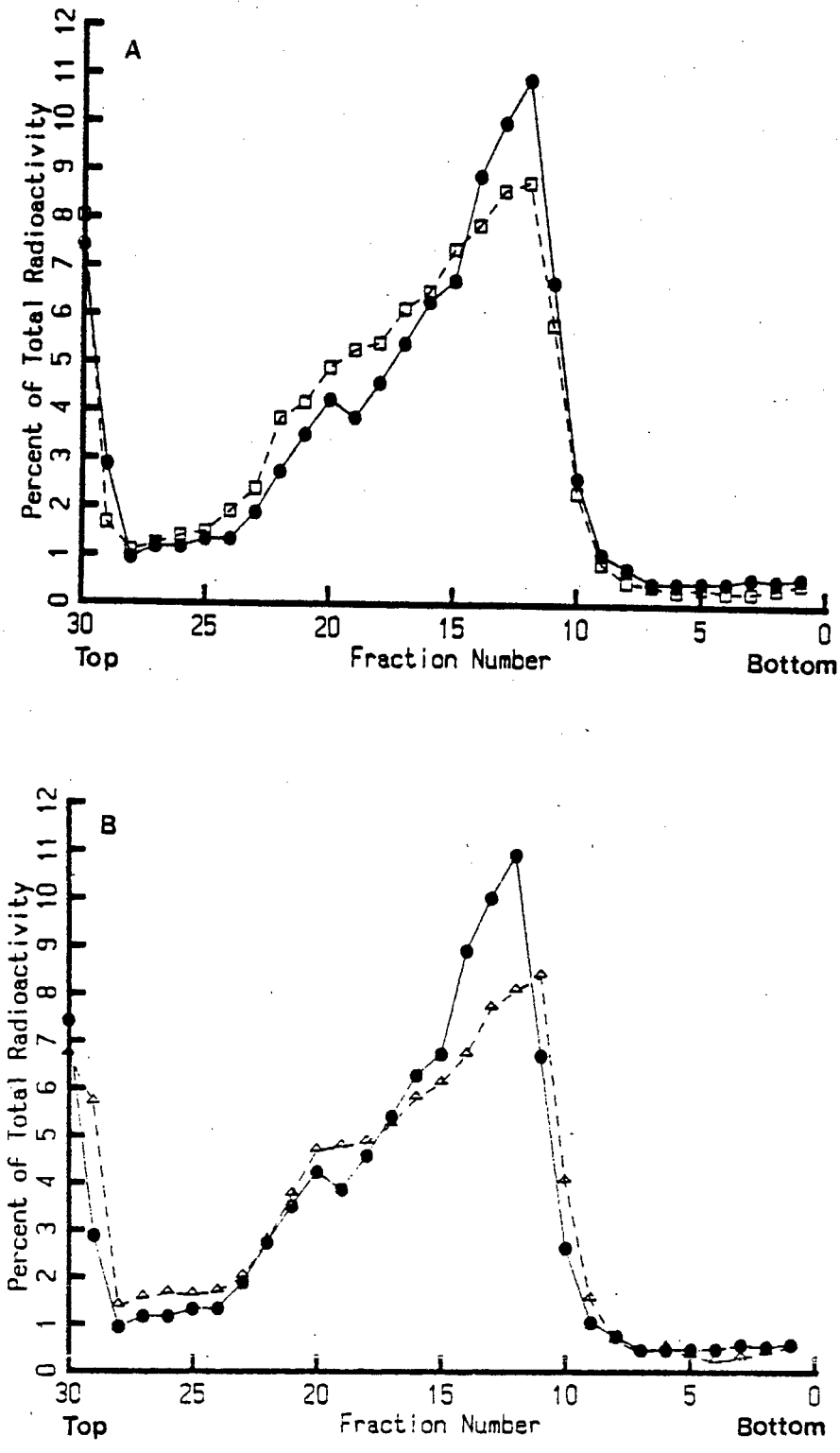



FIGURE 4.9: Alkaline sucrose gradient sedimentation of B.fragilis MTC25 irradiated (15 J $m^{-2}$) and held in aerobic buffer.

A, ●——● control; □- - -□ irradiated, held for 5 min.

B, ●——● control; △- - -△ irradiated, held for 30 min.

| | Holding time (min) | Number of SSB's per $10^8$ daltons wild-type | UVS9 | MTC25 |
|---|---|---|---|---|
| **A** | 0 | 0.01 (0.01) | 0.01 (0.01) | 0.04 (0.01) |
| | 10 | 0.50 (0.15) | 0.29 (0.05) | 0.39 (0.09) |
| | 20 | 0.66 (0.08) | 0.36 (0.05) | 0.35 (0.05) |
| | 40 | 0.91 (0.10) | 0.16 (0.02) | 0.44 (0.06) |
| | 60 | 0.63 (0.06) | 0.08 (0.05) | 0.34 (0.09) |
| | 120 | 0.56 (0.04) | 0.12 (0.06) | 0.21 (0.08) |
| **B** | 0 | 0.08 (0.04) | 0.07 (0.03) | 0.03 (0.02) |
| | 5 | 0.39 (0.05) | 0.19 (0.04) | 0.02 (0.01) |
| | 10 | 0.44 (0.09) | 0.03 (0.02) | 0.18 (0.02) |
| | 15 | 0.26 (0.03) | 0.02 (0.01) | 0.22 (0.03) |
| | 30 | 0.20 (0.01) | NDB | 0.04 (0.02) |
| | 60 | 0.17 (0.12) | | NDB |
| | 90 | NDB | | |
| **C** | 5 | 1.00 (0.20) | | |
| | 20 | 0.41 (0.10) | | |
| | 40 | 0.13 (0.04) | | |
| | 60 | NDB | | |

TABLE 4.2: The number of single-strand breaks (SSB's) accumulated after UV-irradiation (15 J $m^{-2}$) of B.fragilis strains during (A) anaerobic holding in buffer, (B) aerobic holding in buffer, and (C) during replication in minimal medium.
The means of 3 - 5 experiments are given (± the range)
NDB = No detectable breaks.

4.3.2 DNA STRAND-BREAKAGE DURING REPAIR OF UV-IRRADIATED CELLS UNDER REPLICATING CONDITIONS.

Pre-labelled wild-type B.fragilis DNA showed the production of strand-breaks following UV irradiation under replicating conditions. The breaks were generated more rapidly than under holding conditions with a maximum of approximately 1.00 per $10^8$ daltons being reached at 5 min post-irradiation. The $M_n$ of unirradiated DNA was regained at 60 min post-irradiation (Table 4.2C). The $M_n$ of the unirradiated control DNA extracted during replication was found to be higher than that of DNA extracted from non-replicating cells ($170 \times 10^6$ daltons).

4.3.3 THE EFFECT OF CAFFEINE ON THE PRODUCTION OF DNA STRAND-BREAKS

When 2.5 mg $ml^{-1}$ caffeine was added to the irradiation buffer, the percentage transmission of RS for 254 nm UV was decreased from 100% to 0.1%. This would have a major effect on the UV exposure dose administered to the cells, and caffeine was, therefore, added to the experimental system only after irradiation on ice.

The addition of various concentrations of caffeine (0.1 - 2.5 mg $ml^{-1}$) to control, unirradiated cells held at $4^0C$, caused DNA strand-breaks which were observed immediately. These increased in number with increasing caffeine concentration (Fig. 4.10). There was no increase in the number of breaks generated with increasing exposure

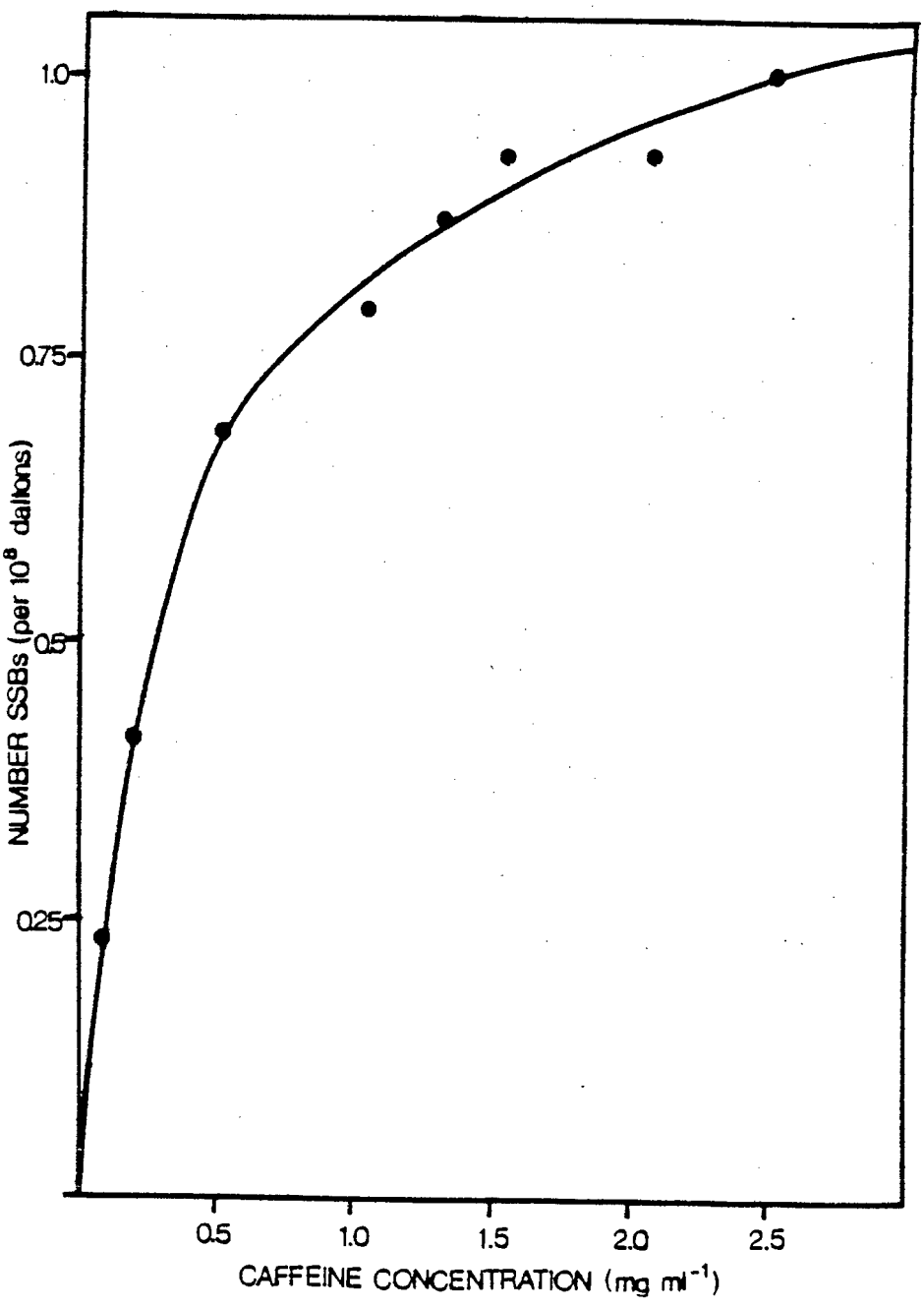



FIGURE 4.10: Induction of single-strand breaks in B.fragilis wild-type DNA treated with caffeine under anaerobic conditions.

time up to 40 min. There was a small decrease in the number of breaks generated under replicating as compared with buffer-holding conditions (0.9 and 1.2 breaks per $10^8$ daltons respectively). Removal of caffeine from the sample before loading on to the gradient did not abolish strand-breakage. This indicates that the breaks are not an artefact of the alkaline gradient conditions interacting with the caffeine and degrading the DNA.

There was no significant difference in the number of breaks caused by caffeine in irradiated or unirradiated DNA held on ice and sampled immediately (Fig. 4.11A,B). Holding of UV-irradiated caffeine-treated cells in buffer at $30^0$C, however, resulted in a dramatic increase in DNA breaks as compared with irradiated cells held without caffeine (Figs 4.12A,B; Table 4.3). These breaks reached an accumulated maximum of approximately 2.6 per $10^8$ daltons but were repaired during holding in the presence of caffeine to the same final level (0.6 SSB per $10^8$ daltons) as the irradiated cells held in buffer alone.

Under replicating conditions, the caffeine-treated and untreated UV-irradiated cells accumulated breaks to the same extent (maximum approximately 1.00 per $10^8$ daltons at 5 min).

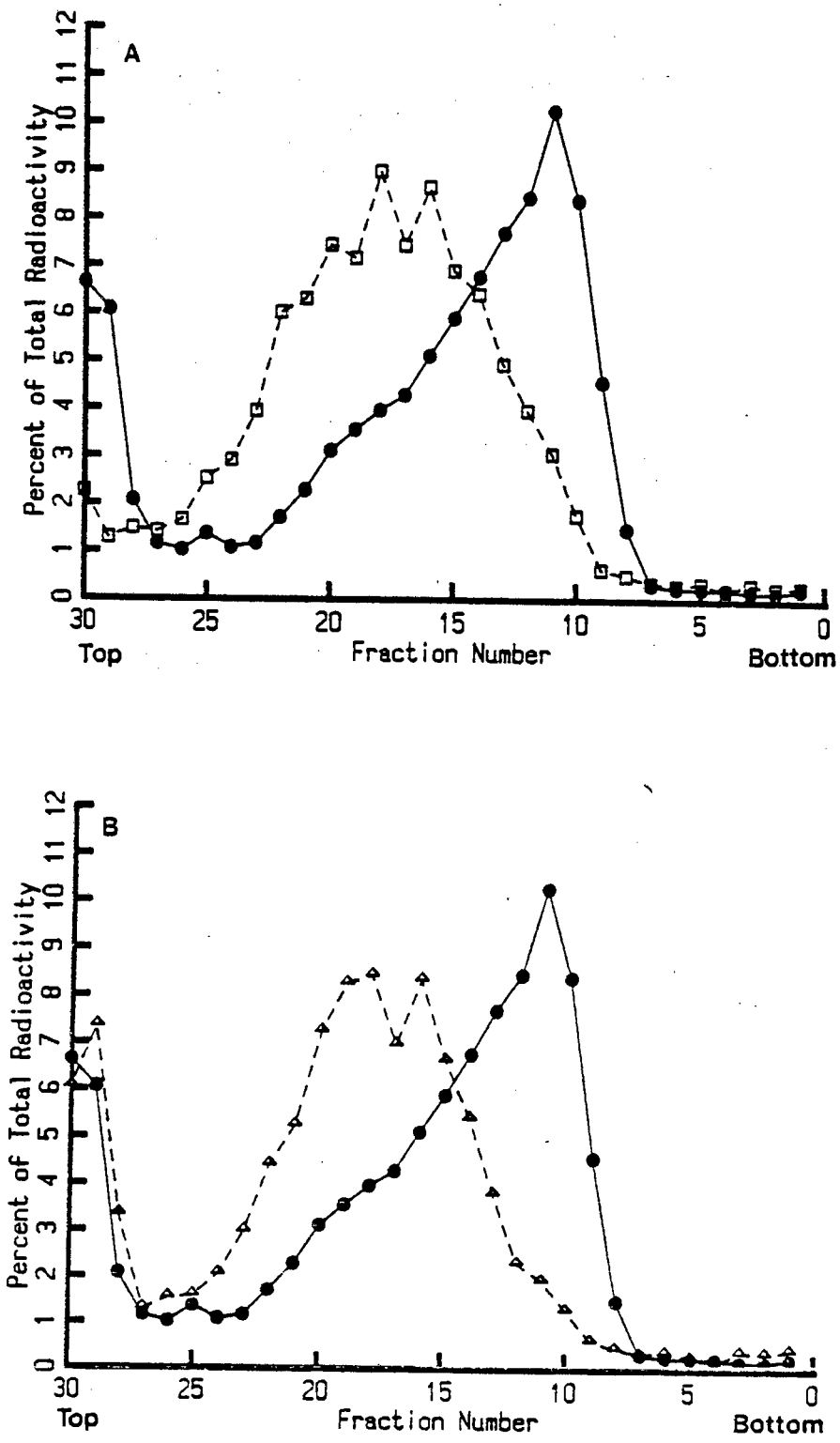



FIGURE 4.11: Alkaline sucrose gradient sedimentation of *B.fragilis* wild-type DNA treated with caffeine (2.5 mg $ml^{-1}$) during anaerobic holding in buffer.

A, unirradiated DNA without ( ●——● )

and with ( □-----□ ) caffeine.

B, irradiated DNA without ( ●——● )

and with ( △----△ ) caffeine.

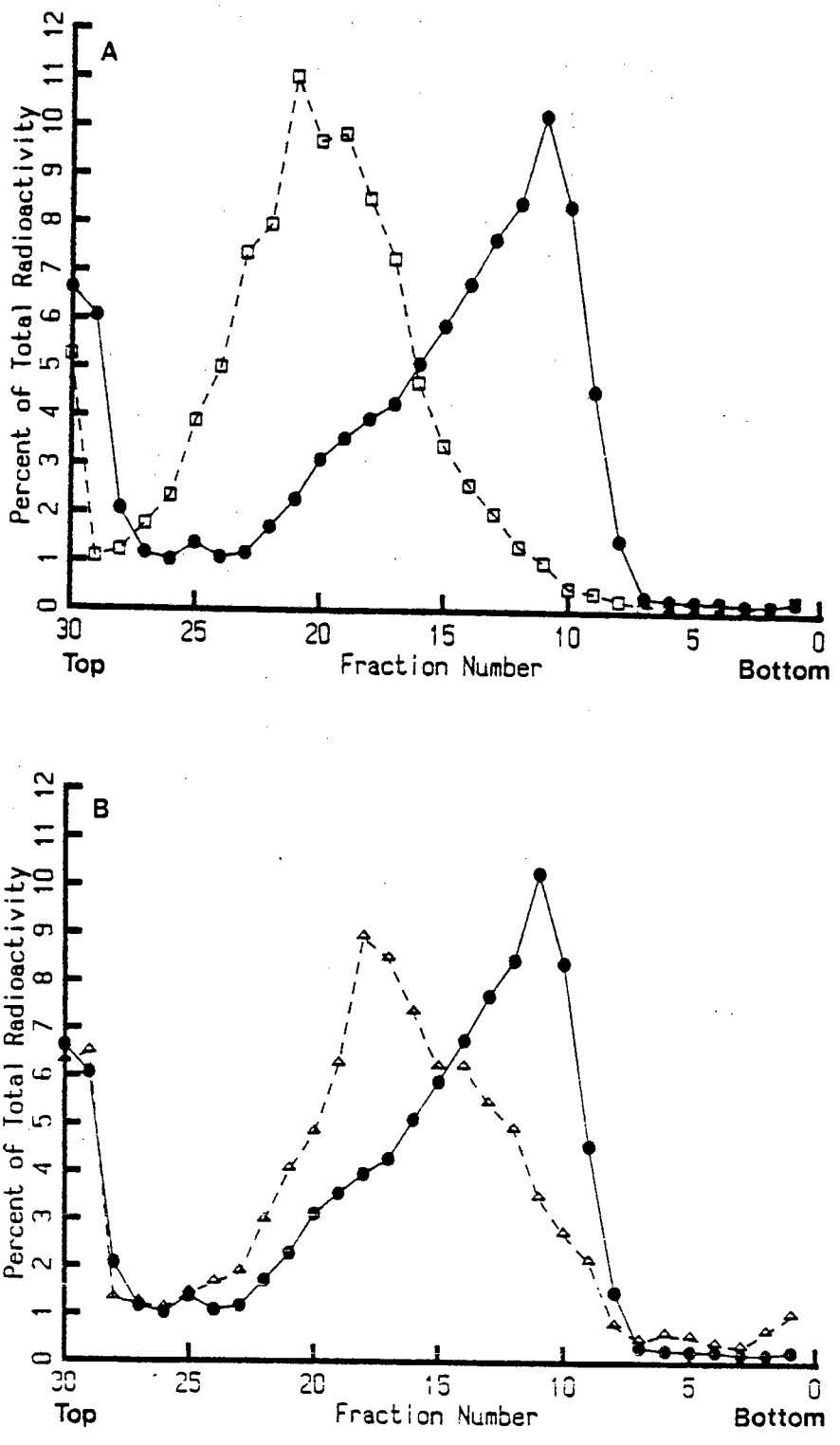



FIGURE 4.12: Alkaline sucrose gradient sedimentation of B.fragilis wild-type, irradiated (15 J $m^{-2}$) and held with caffeine (2.5 mg $ml^{-1}$) under anaerobic conditions.

A, ●—● no caffeine; □- -□ with caffeine, held for 30 min.

B, ●—● no caffeine; △- -△ with caffeine, held for 60 min.

TABLE 4.3:

Effect of caffeine on the number of single-strand breaks $_{-2}$(SSB's) accumulated after UV-irradiation (15 J $m^{-2}$) of *B. fragilis* wild-type during holding in anaerobic buffer.

The means of 3 experiments are shown (± the range).

| Holding Time (min) | Number of SSB's | |
|---|---|---|
| | UV + caff | UV - caff |
| 0 | 0.86 (0.13) | 0.01 (0.01) |
| 15 | 1.76 (0.30) | 0.59 (0.15) |
| 40 | 2.16 (0.40) | 0.91 (0.10) |
| 60 | 0.94 (0.30) | 0.63 (0,06) |
| 90 | 0.63 (0.10) | 0.56 (0.04) |

## 4.3.4 THE EFFECT OF MITOMYCIN C ON B.FRAGILIS DNA

Treatment of the B.fragilis wild-type strain with a lethal dose (1.0 $\mu g\ ml^{-1}$) of mitomycin C under anaerobic conditions caused extensive and progressive loss of TCA precipitable material with exposure time during treatment in buffer at 37°C (Fig. 4.13A). This degradation was completely inhibited by holding on ice (Figs 4.13B). An increase in the molecular weight of the DNA was transiently observed after 5 min exposure at 37°C, but after 15 min virtually total loss of TCA precipitable material was found to have occurred (Fig. 4.13A). Exposure of the wild-type cells to 0.5 $\mu g\ ml^{-1}$ of mitomycin C at 37°C resulted in a marked increase in $M_n$ of the DNA after 3 min and 5 min treatment, with approximately 25% loss of TCA precipitable material (Fig. 4.14).

Treatment of the mutant strains with the lower mitomycin C concentration, however, caused a similar degradative response as had been observed in the wild-type at the higher dose (Figs 4.15; 4.16).

An interesting feature of the degradation response in all three strains was the noticeable persistence of a peak (or peaks) between fractions 16 to 20, before these also underwent degradation during subsequent exposure (Figs 4.13A; 4.14; 4.15; 4.16).

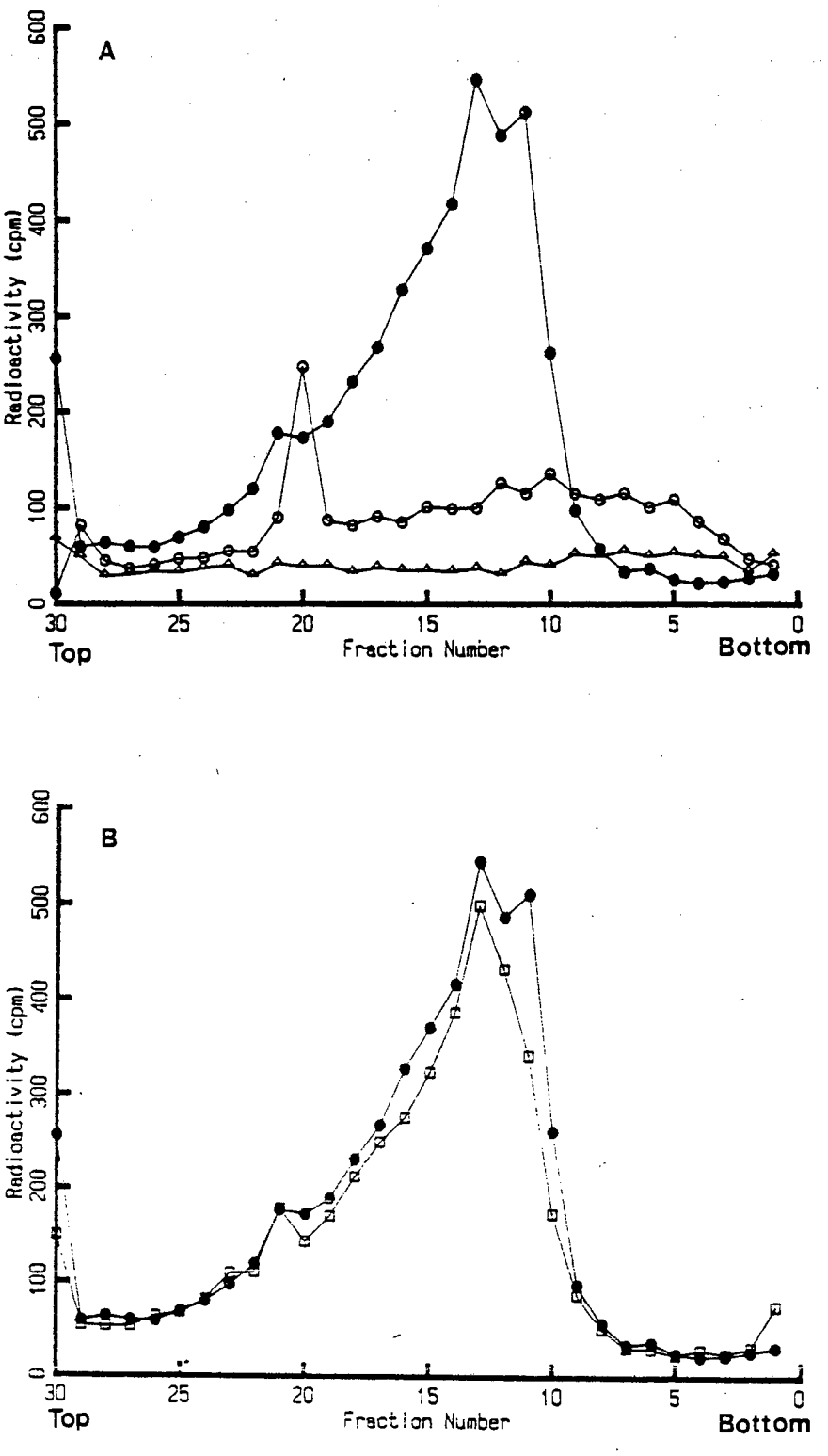



FIGURE 4.13: Alkaline sucrose gradient sedimentation of B.fragilis wild-type treated for various exposure times with mitomycin C (1.0 μg $ml^{-1}$) under anaerobic conditions.

A, treatment at $37^{0}C$;

(●) control; (○) 5 min; (△) 15 min.

B, treatment at $4^{0}C$;

(●) control; (□) 15 min.

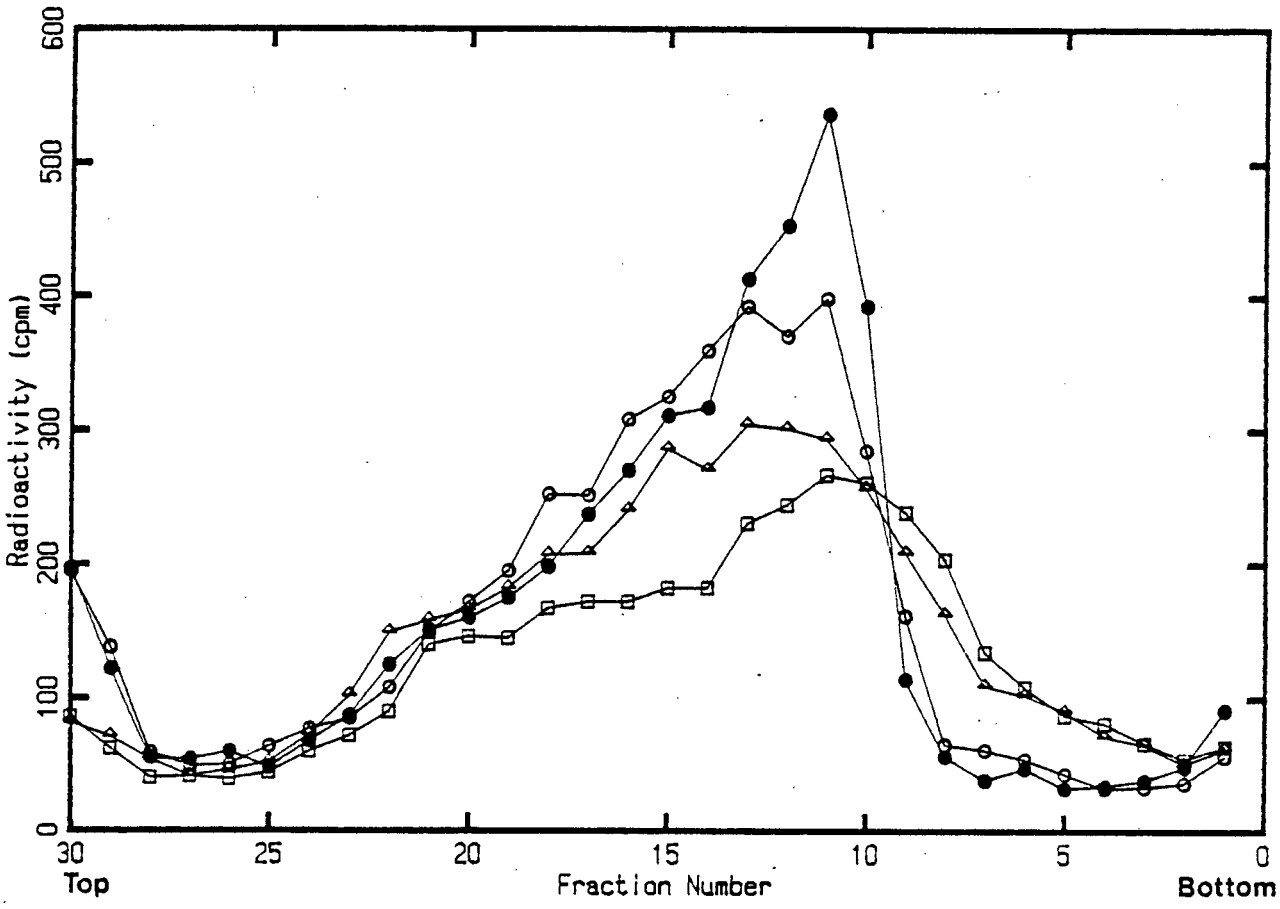



FIGURE 4.14: Alkaline sucrose gradient sedimentation of B.fragilis wild-type treated for various exposure times with mitomycin C (0.5 $\mu g\ ml^{-1}$) under anaerobic conditions. (●) control; (○) 1 min; (△) 3min; (□) 5 min.

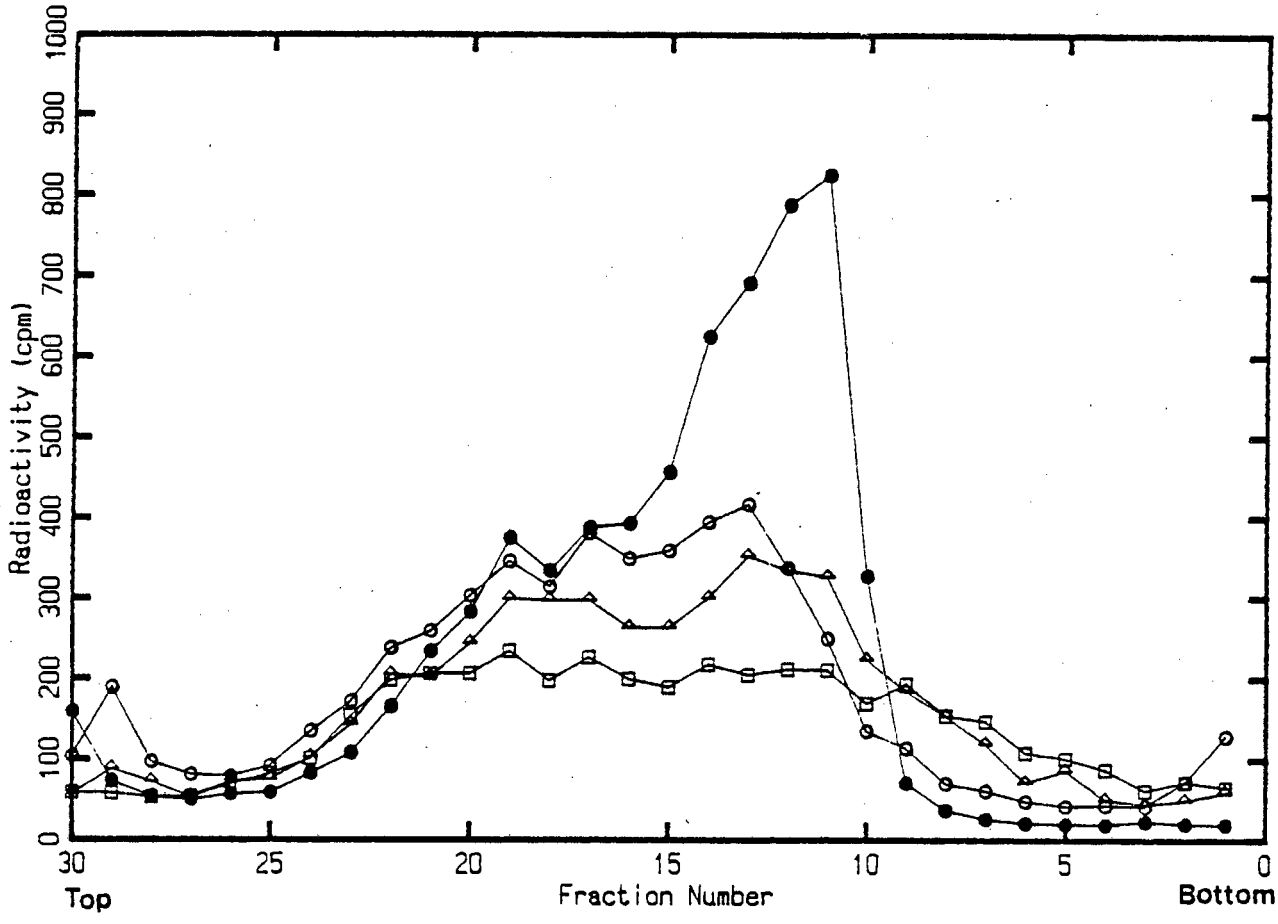



FIGURE 4.15: Alkaline sucrose gradient sedimentation of B.fragilis UVS9 treated for various exposure times with mitomycin C (0.5 $\mu g\ ml^{-1}$) under anaerobic conditions. (●) control; (○) 1 min; (△) 3min; (□) 5 min.

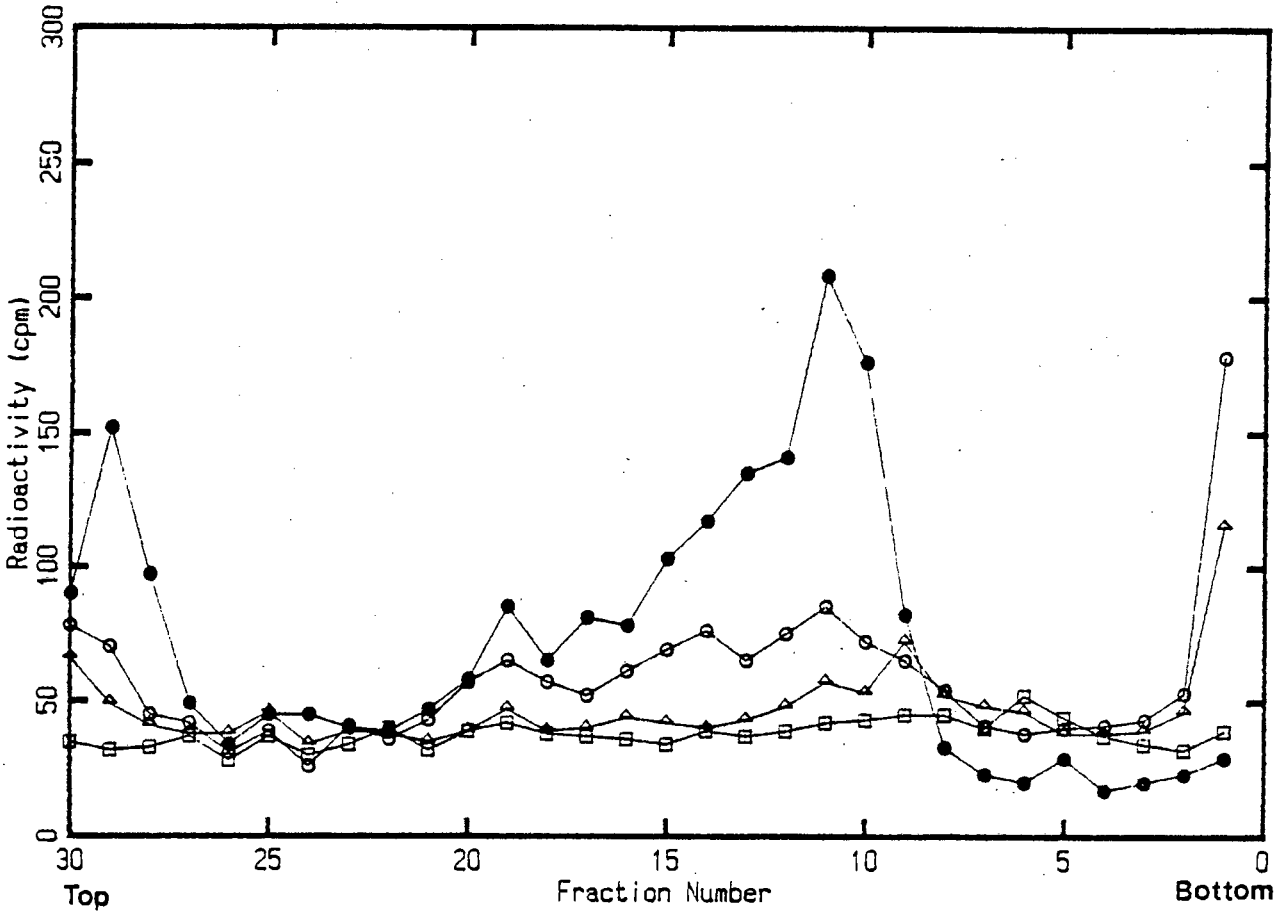



FIGURE 4.16: Alkaline sucrose gradient sedimentation of B.fragilis MTC25 treated for various exposure times with mitomycin C (0.5 μg $ml^{-1}$) under anaerobic conditions. (●) control; (○) 1 min; (△) 3min; (□) 5 min.

## 4.4 DISCUSSION

Alkaline sucrose gradient sedimentation techniques have been used successfully in this work to demonstrate both the direct DNA strand-breakage effect of certain DNA damaging agents, as well as the enzymatic repair responses induced by the damage.

The method used does not enable a distinction to be made between breaks of single- or double-stranded origin, or between breaks generated via alkali-labile sites during exposure to the alkaline gradient conditions. In future studies, sedimentation of the DNA in neutral gradients should enable an assessment of single- or double-stranded breakage (Peak and Peak, 1982).

In this work, the presence of a large plasmid was noted sporadically in the gradient studies (Fig. 4.3) as well as on gels (Chapter III, Fig. 3.4), but displayed variations in copy-number in different experiments. It appeared to have a single-stranded $M_n$ in the 30 - 50 X $10^6$ dalton range. Its contribution to the molecular weight of the DNA could not be assessed independently and it was therefore included in all molecular weight calculations of the B.fragilis chromosomal DNA. This may have introduced a variable into the experimental system since it is not known whether B.fragilis repairs extra-chromosomal elements to the same extent as the chromosomal DNA. White and Sedgewick (1987) have, in fact, reported that in

Saccharomyces cerevisiae, repair of plasmids does not occur during the inducible repair of chromosomal DNA.

Salyers (1985) has reported that plasmids of 100 kb or more have been observed in Bacteroides spp and that their size renders separation from chromosomal DNA being difficult to achieve.

4.4.1 Repair of UV-irradiated DNA

The breaks generated in UV-irradiated B.fragilis DNA were caused by metabolic activity since they were only formed at temperatures where physiological activity was possible. Furthermore, under anaerobic conditions, the number of breaks accumulated by the B.fragilis wild-type strain as compared with the two mutant strains correlated well with the relative efficiency of pyrimidine dimer removal observed in these three strains under the same conditions (Chapter III). It is likely, therefore, that an incision and resynthesis-type repair mechanism contributes to removal of pyrimidine dimers from irradiated B.fragilis DNA.

Complete repair of the breaks was not, however, observed under anaerobic holding conditions. The persistence of approximately 50% of the breaks after 2 h holding could perhaps be the reason for the drop in viability observed in repair proficient wild-type and MTC25 mutant strains during the holding period in spite of effective dimer

removal as detected by the PD-specific enzyme probe (Chapter III).

Under replicating conditions, strand-breaks were generated more rapidly than during holding but there was complete repair of these by 60 min post-irradiation. This result correlates well with the finding of Schumann et al. (1983) that immediately after irradiation under replicating conditions, there is rapid loss of TCA precipitable material from the original pre-labelled DNA. This degradative phase was completed by 60 min post-irradiation.

Aerobic conditions affected the $M_n$ of the B.fragilis DNA by generating strand-breaks. Prolonged exposure to air (longer than 5 h) during lysis on the alkaline gradients enhanced this effect.

Fewer strand-breaks accumulated in the wild-type strain under aerobic than under anaerobic holding conditions, and complete repair of these breaks was achieved within 60 - 90 min. The UVS9 mutant showed very low levels of incision but complete resynthesis of the DNA breaks.

Very few strand-breaks could be detected in the MTC25 mutant. This result is surprising since this mutant shows enhanced loss of PD endonuclease sites (i.e. dimer removal) under these conditions (Chapter III). The implication of this result is that the MTC25 mutant is able to remove dimers by means of a mechanism which does

not involve endonucleolytic incision of the DNA backbone. As was discussed in Chapter I, the only proven mechanism of this type involving removal of pyrimidine dimers is photoreactivation which could not be functioning here under extremely subdued light conditions, and has not been physiologically observed in B.fragilis (Chapter I).

The possibility that some other, as yet unknown, air inducible system exists for the direct removal of pyrimidine dimers in B.fragilis must therefore be considered, perhaps involving a "pyrimidine insertase" mechanism comparable to the purine insertase activity in E.coli described by Livneh et al. (1979) (Chapter I, Section C 1.2).

The process of strand-breakage and repair which has been observed in B.fragilis has the characteristics of an incision, resynthesis and religation system. The maximum number of strand-breaks accumulated at a particular time within the holding period did not approach the much higher level of dimer sites generated by the UV dose administered as observed during in vitro PD-endonuclease digestion. (The actual number of dimers generated by UV in B.fragilis DNA could not be accurately measured due to the technical difficulties of isolating suitably intact chromosomal DNA for quantitative digestion).

The lack of accumulation of high levels of strand-breaks may be interpreted as evidence that the repair in B.fragilis is a dynamic process with strand-breaks not accumulating to any great extent due to concurrent repair synthesis. The dynamics of E.coli excision repair processes have been interpreted in this way by Howard-Flanders (1968).

An alternative explanation could be that the strand-breakage/ resynthesis process observed in these studies was not sufficient to deal with all the dimers generated, and that a concurrent, incisionless system could be operating as well in B.fragilis.

### 4.4.2 The effect of caffeine on B.fragilis DNA

The strand-breaking effect of caffeine on B.fragilis wild-type cells showed that both unirradiated and irradiated DNA were affected to the same extent, and that the breaks were generated directly by the action of the caffeine since inhibition of cellular metabolism at low temperatures did not inhibit strand-breakage. Additional strand-breaks were observed in the irradiated but not the unirradiated DNA under anaerobic holding conditions indicating that strand-breakage induced in response to UV-irradiation damage was not inhibited by the presence of the caffeine. These breaks were repaired to the same final level as in irradiated cells held without caffeine.

It has been shown that under the same experimental conditions, caffeine inhibited the removal of pyrimidine dimers from B.fragilis wild-type DNA (Chapter III). Also, irradiated cells which had been held in the presence of caffeine showed loss in viability compared to caffeine-free controls when plated on a caffeine-free medium, whereas unirradiated cells did not (Chapter III).

The interaction of caffeine with B.fragilis DNA differs in several major respects from its activity reported to date in the literature.

1) The inhibition of the incision capability of the uvrA,B,C endonuclease of E.coli by caffeine has been reviewed in Chapter II. Fong and Bockrath (1979) reported that for complete inhibition of incision to occur, the cells had to be pre-treated with caffeine and irradiated in its presence. The results reported here, however, showed that caffeine in the irradiation buffer absorbed 99.9% of the incident UV light. The lower number of dimers generated by the reduced dose would therefore lead to fewer breaks being observed during repair.
In B.fragilis incision of damaged DNA took place in the presence of caffeine.

2) Caffeine has been proposed as an inhibitor of one of the ATP-requiring enzymes involved in DNA replication, possibly DNA polymerase III (Sandlie et al., 1983).

DNA polymerase III is also thought to be involved in repair resynthesis (Chapter I). In B.fragilis, however, repair resynthesis of DNA strand-breaks occurred in caffeine-treated UV-irradiated cells.

3) The direct strand-breakage effect of caffeine on B.fragilis DNA has not been observed in the majority of prokaryotic cells (Reviewed by Timson, 1977). It has, however, been reported that caffeine increased the rate of spontaneous DNA breakdown during normal growth of E.coli Bs-11, but not in the parental B strain (Grigg, 1970).

Chromosome abnormalities and breakage have been observed in eukaryotic cells as a major effect of caffeine treatment (Timson, 1977).

The available results suggest two possible levels for the enhanced lethal effect of caffeine on UV-irradiated B.fragilis cells.

If the observed strand-breaks occurring in UV-irradiated DNA during holding reflect an incision, excision, and resynthesis mode of repair, then caffeine does not inhibit this system in B.fragilis and must be inhibiting some other type of UV repair system. Livneh et al. (1979) showed that the purine insertase system of E.coli was inhibited by caffeine.

Alternatively, the effect of the caffeine may be exerted directly at the DNA level. The accumulation of high levels of DNA strand-breaks resulting from the additive effect of both caffeine and repair enzyme activity may have a lethal effect on the cell even though these breaks are repaired to the same final levels as UV-induced repair breaks alone.

4.4.3 Effect of mitomycin C on B.fragilis DNA

The experiments reported here show that mitomycin C was only able to interact with B.fragilis DNA under physiologically active conditions. This is in agreement with the reported activation of mitomycin C through enzymatic reduction in vivo (Szybalski and Iyer, 1967).

DNA cross-linking was caused by treatment of B.fragilis DNA with lethal doses of mitomycin C. However, there was a pronounced degradative effect as well which seemed to depend on the individual level of sensitivity of the various strains to mitomycin C. Investigation of the behaviour of these strains at concentrations closer to their individual MICs could perhaps overcome the degradation phenomenon so that their respective cross-link and repair capacities could be investigated.

Kersten (1971) has reviewed the phenomenon of DNA degradation in response to mitomycin C treatment and has pointed out that it is only rarely found in bacterial systems. The degradation observed in this work on

B.fragilis was, however, a major effect of mitomycin C treatment at lethal doses and the loss of TCA precipitable material would indicate degradation to the level of very short nucleotide chains or even bases. The degradation phenomenon could probably be studied more exactly by measuring the loss of TCA precipitable material from pre-labelled DNA treated with mitomycin C rather than by means of alkaline gradient analysis.

The persistence of DNA peaks in the mitomycin C- degraded gradient profiles at the positions thought to be those of the large plasmid(s) carried by this B.fragilis strain was noted. An increase in resistance of plasmid, viral and episomal factors to mitomycin C degradation has been reported previously in several other experimental systems. Driskell-Zamenhof and Adelberg (1963) reported that the $F^+$ factor in E.coli cells was more resistant to degradation than the DNA of the host cell, and Szybalski and Iyer (1967) have reviewed this phenomenon in various host/virus situations.

The effect of mitomycin C treatment in enhancing a secondary peak of B.fragilis DNA could, alternatively, be interpreted as the induction of a viral or episomal factor off the chromosome. Previous efforts to detect lysogenic phage in B.fragilis have been unsuccessful (Reviewed in Chapter I). The experimental conditions used in this work could, perhaps, be applied in further investigations in this direction.

# CHAPTER V

# DISCUSSION

The results reported in this thesis indicate that B.fragilis strain Bf-2 shows elements of a UV repair system involving incision, excision and resynthesis of DNA during dimer removal. Evidence for some other, as yet unknown, cellular repair response is also present.

The isolation of the first stable repair deficient mutants of B.fragilis has provided a basis for the examination of DNA repair systems in this organism at the physiological and molecular levels. More mutants of this type would be of value in extending these studies further. In particular, a totally incisionless mutant would be necessary for assessing post-replication repair, and also the possible presence of a direct dimer removal system.

The isolation of mutants of this type could be achieved through mutagenesis of the wild-type strain. Alternatively, it may be necessary to form double mutants of the existing UVS9 and MTC25 strains if two separate pyrimidine dimer and mitomycin C repair pathways exsist as is the case in M.radiodurans (Evans and Moseley, 1983; Moseley and Evans, 1983).

The mutability of B.fragilis with EMS has been established in this study. It would be of interest to examine this organism for the presence of a umu-like gene comparable to

the umuDC genes of E.coli (Walker et al., 1982; Elledge and Walker, 1983), and the umu-like gene of S.typhimurium (Orrego and Eisenstadt, 1987) which are found to be necessary for mutation through cellular SOS processing of damaged DNA.

The presence of such a gene could be detected through cloning of a B.fragilis gene bank into an E.coli umu mutant which is also mutant in another selectable gene (e.g. an amino acid) and looking for damage-induced reversion of the marker gene.

Alternatively, an attempt could be made to clone a plasmid such as pKM101 into B.fragilis. This plasmid carries analogues of the umu genes (mucA and mucB) which have been found to increase the mutability of the strain into which they are introduced if the transformed strain does not have its own umu system, or if it is functioning at levels less than that coded for by the plasmid genes (Mortelmans and Stocker, 1979).

Cloning of B.fragilis repair genes into known E.coli mutants would also be of value in establishing the mechanisms and regulation of these genes. The ultimate objective of these studies would be complementation studies between cloned genes in B.fragilis mutants using the recently developed transformation or shuttle-plasmid genetic transfer techniques.

A gene bank of B.fragilis strain 01 was established by Southern (1986) from which Goodman et al. (In press) have cloned a recA-like gene. The work described in this thesis was conducted on strain Bf-2 and a gene bank of this strain has recently been made using pEcoR251 cloning into E.coli DK1 (personal work in progress) . To date, a putative recA-like clone has been isolated which increased the UV resistance of both E.coli DK1 and CSR603 recA mutant strains. Screening is continuing for uvr-like genes.

Studies on B.fragilis have shown the induction of several proteins in response to physiological stress (Reviewed in Chapter I). Functional roles have not yet been assigned to these proteins. An investigation of the enzymatic activities of these proteins would be of interest in establishing the parts played by them in cellular protection from stress or in repair.

A starting point for such a study would be the assessment of the roles played by hydrogen peroxide and superoxide radical scavangers such superoxide dismutase, catalase and peroxidase enzymes, and whether any of the observed proteins induced by oxygen-related stress are, in fact, these enzymes. Sensitive activity gel assay methods are available for demonstrating the activities of these enzymes (Gregory and Fridovich, 1974; Liu and Gibson, 1977; Beauchamp and Fridovich, 1971). It would,

therefore, be possible to make a direct comparison between the observed induction of a particular protein and its activity.

Cloning of the genes coding for these enzymes would also be of interest in establishing the existence of stress-induced regulation. There have been reports in the literature that oxygen has been found to induce higher levels of catalase and peroxidase (Hassan and Friedovich, 1977) and superoxide dismutase (Gregory et al., 1977; Tally et al., 1977) in B.fragilis.

Techniques for the isolation of large plasmids are, in general, limited in value due to chromosomal DNA interference, and to date pulse electrophoresis is reported as being the most successful (Carle et al., 1986).

An interesting finding emerging from this study is the DNA degradation response shown by B.fragilis after treatment with mitomycin C and the relative resistance of the large plasmid to this degradation. There is also apparently an increase in molecular weight of the chromosomal DNA relative to the plasmid under alkaline conditions due to mitomycin C-induced cross-linkage in the former. It is possible that this differential response could be applied to the isolation of large plasmids from B.fragilis.

# APPENDIX 1

## MEDIA AND CHEMICAL REAGENTS

### 1 PREPARATION OF ANAEROBIC MEDIA AND SOLUTIONS

Pre-reduced and anaerobically sterilized media and solutions were prepared according to the methods of Moore (1966), Hungate (1969) and Holdeman and Moore (1969). Most of the oxygen in media was driven off by heating, and the media were further reduced by the addition of cysteine hydrochloride or sodium thioglycollate. They were then flushed with $CO_2$ and maintained in an anaerobic state in Astell roll tubes with tightly fitting rubber stoppers, Hungate tubes, or in anaerobic jars or boxes containing silica gel.

All sterilization was done by autoclaving at 121°C for 15 min unless otherwise stated.

### 2 STOCK SOLUTIONS

### 2.1 CYSTEINE STOCK SOLUTION

| | |
|---|---|
| Cysteine hydrochloride | 1.0 g |
| Distilled $H_2O$ | 20.0 ml |

1 ml autoclaved cysteine stock solution was added per 100 ml cooled medium prior to pouring to give a final concentration of 0.5 mg $ml^{-1}$.

2.2 SODIUM CARBONATE STOCK SOLUTION

| | |
|---|---|
| $Na_2CO_3$ | 20.0 g |
| Distilled $H_2O$ | 100.0 ml |

Autoclaved.

2 ml of $Na_2CO_3$ stock was added per 100 ml medium to give a final concentration of 4 mg $ml^{-1}$.

2.3 HAEMIN-MENADIONE STOCK SOLUTION

2.3.1 Menadione Stock Solution

| | |
|---|---|
| Menadione | 100.0 mg |
| 95% ethanol | 20.0 ml |

Filter-sterilized and stored at 4°C.

2.3.2 Haemin Stock Solution

| | |
|---|---|
| Haemin | 50.0 mg |
| 1 N NaOH | 1.0 ml |
| Distilled $H_2O$ | 100.0 ml |

Autoclaved and stored at 4°C.

2.3.3 Haemin-Menadione Stock Solution

| | |
|---|---|
| Menadione Stock Solution | 1.0 ml |
| Haemin Stock Solution | 100.0 ml |

1 ml of Haemin-Menadione stock solution was added per 100 ml cooled medium to give a final concentration of 5 $\mu g\ ml^{-1}$.

2.4 RESAZURIN STOCK

| | |
|---|---|
| Resazurin | 25.0 mg |
| Distilled $H_2O$ | 100.0 ml |

3 BUFFERS

3.1 T2 BUFFER

Solution A :

| | |
|---|---|
| $KH_2PO_4$ | 7.5 g |
| NaCl | 20.0 g |
| $NA_2HPO_4$ (anhydrous) | 15.0 g |
| $K_2SO_4$ | 25.0 g |
| $CaCl_2$ * | 0.055 g |
| Distilled $H_2O$ | 1 000.0 ml |

Stored over 10% chloroform.

* Make up separately in a little distilled water first.

Solution B:

| | |
|---|---|
| $MgSO_4$ | 4.8 g |
| Distilled $H_2O$ | 100.0 ml |

Autoclaved.

Solution C:

| | |
|---|---|
| Gelatin | 0.2 g |
| Distilled $H_2O$ | 100.0 ml |

Autoclaved.

To make up:

| | |
|---|---|
| Solution A | 40.0 ml |
| Distilled $H_2O$ | 158.0 ml |
| Solution B | 1.0 ml |
| Solution C | 1.0 ml |

Autoclaved.

3.2 RINGER SOLUTION (Quarter strength)

| | |
|---|---|
| NaCl | 2.25 g |
| KCl | 0.105 g |
| $CaCl_2$ | 0.12 g |
| $NaHCO_3$ | 0.05 g |
| Distilled $H_2O$ | 1 000.0 ml |

Autoclaved. For anaerobic use, the solution was steamed for 30 min, gassed and allowed to stand in the anaerobic cabinet for 1 - 2 days.

3.3 PHOSPHATE BUFFER (FOR E. coli)

Solution A:

| | |
|---|---|
| $Na_2HPO_4.12\ H_2O$ | 7.0 g |
| $KH_2PO_4$ | 3.0 g |
| NaCl | 4.0 g |
| $H_2O$ | 1 000.0 ml |

Autoclaved.

Solution B:

0.5 M $MgSO_4$ Stock

| | |
|---|---|
| $MgSO_4$ | 12.32 g |
| $H_2O$ | 100.0 ml |

Autoclaved.

Add 4 ml $MgSO_4$ stock to Solution A after autoclaving.

3.4 TBE BUFFER (10 x STOCK)

| | |
|---|---|
| Tris | 108.0 g |
| Boric Acid | 55.0 g |
| $Na_2EDTA.2H_2O$ | 9.3 g |
| Distilled $H_2O$ | 1 000.0 ml |

Dilute 10 x before use.

4 ANAEROBIC MEDIA

4.1 BRAIN HEART INFUSION BROTH

| | |
|---|---|
| BHI (Difco) | 18.5 g |
| Yeast extract | 2.5 g |
| Sodium thioglycollate | 0.55 g |
| $Na_2CO_3$ | 2.0 g |
| Distilled $H_2O$ | 500.0 ml |
| Resazurin stock solution | 1.0 ml |

The medium was steamed for 30 min and 5.0 ml Haemin-Menadione stock solution was added. The broth was dispensed into Hungate tubes in 10 ml volumes, gassed with $CO_2$ and autoclaved.

4.2 BRAIN HEART INFUSION AGAR

4.2.1 Plates

| | |
|---|---|
| BHI (Difco) | 18.5 g |
| Yeast Extract | 2.5 g |
| Agar | 7.5 g |
| Distilled $H_2O$ | 500.0 ml |

The medium was autoclaved, cooled and the following were added:

| | |
|---|---|
| cysteine stock solution | 5.0 ml |
| $Na_2CO_3$ stock solution | 10.0 ml |
| Haemin-Menadione stock solution | 5.0 ml |

4.2.2 Slopes

| | |
|---|---|
| BHI (Difco) | 18.5 g |
| Yeast extract | 2.5 g |
| Agar | 7.5 g |
| Distilled $H_2O$ | 500.0 ml |

The medium was steamed for 30 min and the following were added:

| | |
|---|---|
| Cysteine stock solution | 5.0 ml |
| $Na_2CO_3$ stock solution | 10.0 ml |
| Haemin-Menadione stock solution | 5.0 ml |

The medium was dispensed in 10 ml amounts in Hungate tubes, gassed, capped, autoclaved and sloped.

4.2.3 Soft Agar

| | |
|---|---|
| BHI | 3.7 g |
| Yeast extract | 0.5 g |
| $Na_2CO_3$ | 0.4 g |
| Agar | 0.7 g |
| Distilled $H_2O$ | 500.0 ml |

The medium was autoclaved and stored aerobically. It was steamed for 30 min prior to use, cooled to 50°C while being flushed with $CO_2$ and 5 ml Haemin-Menadione stock solution was added. For aerobic plating experiments the steamed solution was not flushed with $CO_2$.

## 4.3 MINIMAL MEDIUM BROTH

Solutions for Minimal Medium Broth

### 4.3.1 Mineral Solution

| | |
|---|---|
| $KH_2PO_4$ | 18.0 g |
| NaCl | 18.0 g |
| $CaCl_2.2H_2O$ | 0.53 g |
| $MgCl_2.6H_2O$ | 0.4 g |
| $MnCl_2.4H_2O$ | 0.2 g |
| $CoCl_2.6H_2O$ | 0.02 g |

Made up to 1 000 ml with 2 x glass distilled $H_2O$. The salts were added in the order given, and each dissolved completely before adding the next chemical. Stored over 10% chloroform at 4°C.

### 4.3.2 $FeSO_4.7H_2O$ Stock Solution

| | |
|---|---|
| $FeSO_4.7H_2O$ | 0.04 g |
| Distilled $H_2O$ | 100.0 ml |

Stored over 10% chloroform at 4°C.

### 4.3.3 $NH_4Cl$ Stock Solution

| | |
|---|---|
| $NH_4Cl$ | 1.272 g |
| Distilled $H_2O$ | 200.0 ml |

Autoclaved.

4.3.4 Glucose Stock Solution

| | |
|---|---|
| Glucose | 10.0 g |
| Distilled $H_2O$ | 100.0 ml |

Autoclaved.

4.3.5 Vitamin B12 Stock Solution

A stock solution of 1 $\mu$g $ml^{-1}$ was made up with sterile distilled water and filter sterilized. Stored at 4°C.

4.3.6 5% Cysteine Stock Solution

As in 2.1

4.3.7 20% Sodium Carbonate Stock

As in 2.2

4.3.8 Haemin-Menadione Stock

As in 2.3

4.3.9 Minimal Medium Broth

| | |
|---|---|
| Mineral solution | 25.0 ml |
| $FeSO_4.7H_2O$ stock solution | 5.0 ml |
| $NH_4Cl$ Stock Solution | 25.0 ml |
| Resazurin Stock Solution | 1.0 ml |
| Distilled $H_2O$ | 398.5 ml |

pH to 6.5 with NaOH before autoclaving.

Immediately after autoclaving, the following hot solutions were added asceptically while gassing with $CO_2$:

| | |
|---|---|
| Glucose Stock Solution | 25.0 ml |
| $Na_2CO_3$ Stock Solution | 10.0 ml |
| Cysteine Stock Solution | 5.0 ml |
| Haemin-Menadione Stock Solution | 5.0 ml |
| Vitamin B12 Stock Solution | 0.5 ml |

The broth was dispensed asceptically into sterile pre-gassed Hungate tubes in 10 ml amounts with continuous gassing and was stored in the dark anaerobically.

## 5 AEROBIC MEDIA

### 5.1 LURIA BROTH

| | |
|---|---|
| Tryptone | 5.0 g |
| Yeast Extract | 2.5 g |
| NaCl | 5.0 g |
| Distilled $H_2O$ | 500.0 ml |

Autoclave.

### 5.2 LURIA AGAR

| | |
|---|---|
| Luria Broth | 1 000.0 ml |
| Agar | 15.0 g |

## 6 ALKALINE AGAROSE GEL ELECTROPHORESIS

Addition of NaOH to a hot agarose solution causes hydrolysis of the polymer and inability to solidify. The 0.5% agarose gel was, therefore, prepared in a neutral unbuffered solution and equilibrated in the alkaline electrophoresis buffer for 1 h before running.

6.1 NEUTRAL BUFFER

| Final Concentration | | |
|---|---|---|
| 50 mM NaCl | | 2.92 g |
| 1 mM EDTA | (of 0.5 M Stock) | 2.00 ml |
| Distilled $H_2O$ | | 1 000.0 ml |

6.2 ALKALINE ELECTROPHORESIS BUFFER

| Final Concentration | | |
|---|---|---|
| 30 mM NaOH | | 1.2 g |
| 2 mM EDTA | (of 0.5 M Stock) | 4.0 ml |

6.3 ALKALINE TRACKING DYE

| Final Concentration | | |
|---|---|---|
| 50 mM NaOH | (of 0.5 M Stock) | 1.0 ml |
| 25% glycerol | | 2.5 ml |
| 0.12% bromocresol green | | 0.012 g |

Made up to 10 ml with distilled $H_2O$.

7 ALKALINE GRADIENTS

7.1 ALKALINE SUCROSE SOLUTIONS

| | 5% | 20% |
|---|---|---|
| NaCl | 20.5 g | 20.5 g |
| EDTA | 1.86 g | 1.86 g |
| NaOH | 6.0 g | 6.0 g |
| Sucrose (DNase/RNase-free) | 25.0 g | 100.0 g |

Made up to 500 ml with sterile distilled $H_2O$.

Refractive index adjusted to 5% - 1.3493; 20% - 1.3706.

## 7.2 ALKALINE LYSIS BUFFER FOR SUCROSE GRADIENTS

As for Alkaline Sucrose Solutions but excluding the sucrose.

Autoclaved. SDS added to final concentration 0.1% immediately before use.

## APPENDIX II

### MOLECULAR WEIGHT CALCULATIONS

The average molecular weights used in the calculations in Chapter IV are the number-average molecular weights. They were calculated using the following equations:-

Molecular weights were ascribed to each fraction of the gradient by means of the formula

$$MW = \alpha \times (D/\omega^2 t)^{2.62}$$

where

D = fractional distance travelled on the gradient.

$\omega$ = speed in rpm ($\times 10^3$).

t = time in min.

$\alpha$ = $7.33 \times 10^7$ (for a 5 - 20% gradient, calibrated with phage T4 DNA).

To calculate D for a particular fraction (number i):-

$$D_i = N + 0.5 - (\text{Fraction number } i)/N$$

where N is the total number of fractions in the gradient.

The distribution of radioactivity in the gradient was then related to the molecular weights of the various fractions and the average number-molecular weights ($M_n$) calculated.

$$\text{Average } M_n \ (\times 10^6) = \Sigma R_i \ / \ \Sigma (R_i/M_i) \text{ daltons}$$

where $R_i$ is the radioactivity in the ith fraction and $M_i$ is the molecular weight of DNA in the ith fraction.

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