GENETIC STUDIES AND PHYSIOLOGICAL RESPONSES TO ULTRAVIOLET RADIATION IN THE *BACTEROIDES FRAGILIS* GROUP

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF SCIENCE,
RHODES UNIVERSITY, GRAHAMSTOWN

BY

DAVID TODMAN JONES

DECEMBER, 1979

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Professor David R. Woods, for his encouragement, enthusiasm and guidance throughout this study. I also wish to acknowledge my indebtedness to my wife Wyn who helped greatly, both with technical assistance and with the preparation of this manuscript. Without her help, support and encouragement this task could not have been completed in the time available.

In addition, I would like to thank Dr Meyrick Peak for help and advice generously given, and I am grateful to fellow anaerobe workers, Dr Shez Burt for blazing the trail and Kevin Mossie for his cheerful and unselfish approach which made working together a pleasure.

Thanks are also offered to the other members of the department for their interest and suggestions and to Heather Kew and Mrs Yvonne White for typing the manuscript.

CONTENTS

PART I

GENETIC STUDIES ON THE *BACTEROIDES FRAGILIS* GROUP

PART II

PHYSIOLOGICAL RESPONSES OF *B.FRAGILIS* TO ULTRAVIOLET RADIATION

ABSTRACT

The contents of this thesis have been divided into 2 parts. The first part deals with genetic studies carried out on 36 strains belonging to the *Bacteroides fragilis* group. A number of mutants were isolated from several of the strains. A notable feature of the methods used was the low yield of mutants obtained and the marked sensitivity of these organisms to the mutagenic agents. Variations in colonial morphology was found to be a common feature amongst these organisms. In a few strains this phenomenon was clearly visible, in the remainder it was much weaker, and often could only be seen with the aid of a microscope. Colonial variation was found to be due to the ability of a proportion of the cells to pruduce capsules or slime layers. The variants were found to segregate at high frequency and different growth conditions were found to have little effect on the segregation frequency or capsule formation. A number of phages specific for *B.fragilis* and *B.thetaiotaomicron* were isolated. All these phages were virulent and attempts to induce lysogenic phages were unsuccesful. The use of these phages in attempts to obtain transduction proved unsuccessful. A phage carrier state was found to occur in the majority of the phage-host cell systems, which seemed to be due to the presence of phage-resistant encapsulated cells in the population. Bacteriocins were produced by about half the strains, these inhibited the growth of a high proportion of the 36 strains tested. The bacteriocins were released into the growth media at the end of the growth period in the 2 bacteriocins tested. A link between the mode of action of one bacteriocin and rifampicin-resistance was investigated. All the bacteriocins tested were found to be inactive against some rifampicin-resistant mutants of a susceptible strain, suggesting a common mode of action. The presence of capsules in some cells appeared to confer bacteriocin-resistance on these variants.

The second part of the thesis deals with a study of the physiological responses of a single strain of *B.fragilis* to ultraviolet radiation. This strain was found to be more sensitive to ultraviolet radiation under aerobic conditions. The amount of pyrimidine dimers formed after irradiation under anaerobic and aerobic conditions, was not found to differ significantly, indicating that the increase in sensitivity under aerobic conditions was not due to an increase in DNA damage. The use of repair inhibitors and the survival characteristics indicate that this difference was due to decreased repair capabilities under aerobic conditions. Liquid holding recovery in *B.fragilis* was found to occur under aerobic conditions. This process was brought about by excision repair and appeared to be due to a decrease in repair efficiency under aerobic conditions. Under anaerobic conditions, where full repair capabilities were present, liquid holding recovery was inhibited. Both minimal medium recovery and fluence dependent filament formation were found to occur in irradiated *B.fragilis* cells.

The survival kinetics of a number of irradiated *B.fragilis* phages were determined and a number of phage reactivation processes were investigated. Little or no host cell reactivation appeared to occur in the strains investigated, however, some ultraviolet reactivation and multiplicity reactivation was found to occur, but only under anaerobic conditions. Photoreactivation was found to be absent in this organism, but an excision repair system was present. The excision repair system was partially characterized and was found to resemble short patch excision repair in

E.coli. Evidence was found which suggested that a second mode of repair which was sensitive to oxygen, also occurred in this strain. This repair system which appeared to be responsible for error-prone repair, and the systems which were responsible for ultraviolet reactivation and multiplicity reactivation, seemed to be dependent on a 'recombination function' which was inhibited by oxygen. The significance of this finding for future genetic studies was discussed.

CHAPTER I

GENERAL INTRODUCTION

The existence of anaerobic bacteria was discovered by Pasteur just over a hundred years ago, and it was he who introduced the terms aerobic and anaerobic to designate microbes which were able to grow in the presence and absence of oxygen. Anaerobic bacteria comprise a large and diverse assemblage of forms, which include both autotrophs and heterotrophs. Present day evidence indicates that much of the early phase of biological evolution occurred in a world which was totally anaerobic, and that the present oxygen containing biosphere has been a relatively late development, resulting as a consequence of photosynthesis. It seems probable that the majority of extant anaerobic prokaryotic groups are directly descended from the early anaerobic forms. Oxygen is exceedingly useful both in bioenergetic and biosynthetic metabolism, but organisms that utilize oxygen must maintain defences against its deleterious effects. Oxygen is toxic to organisms mainly because of the reactivity of the intermediates and the entities that may be generated by these active products. The topic of oxygen toxicity has been reviewed by O'Brien & Morris (1971), Morris (1975 & 1976) and Fridovich (1972, 1975 & 1977) and the following information has been obtained from these reviews.

Oxygen most commonly exists in the inactivated ground state which is called triplet oxygen ($^3O_2$). Singlet oxygen is a higher energy form of oxygen formed when the two outer electrons achieve antiparallel spins, either in the same orbitals or in separate orbitals (Morris

1975, 1976). Singlet oxygen is extremely reactive and is one of the forms of oxygen that is toxic to living organisms. It may be produced chemically in a variety of ways and is an atmospheric pollutant in smog. Singlet oxygen may also be produced biochemically through the action of certain enzyme systems or the action of visible light. The reduction of O_2 to H_2O which occurs during respiration requires the addition of four electrons. This reduction usually occurs by single electron steps and the first product formed in the reduction of O_2 is the superoxide anion O_2^-. Flavines, flavoproteins, quinones, thiols, and iron-sulphur proteins all carry out one-electron reductions of oxygen to superoxide. This intermediate which is probably produced transiently in small amounts during normal respiratory processes, is highly reactive and can cause oxidative destruction of lipids and other biochemical components. The next intermediate in the reduction of oxygen is peroxide, which is formed biochemically during respiratory processes by a two-electron reduction of O_2, generally mediated by flavoproteins. It is probably produced in small amounts by almost all organisms growing aerobically. Hydroxyl free radical $OH^\bullet$ is the most reactive of the various oxygen intermediates. It is the most potent oxidizing agent known and is capable of attacking any of the organic substances present in cells. Hydroxyl radical is formed as a result of the action of ionizing radiation, and can also be produced chemically via a reaction (mediated by ferric iron) between superoxide and peroxide $O_2^- + H_2O_2 \longrightarrow OH^- + OH^\bullet + O_2$. Thus in any living system in which superoxide and peroxide are generated simultaneously, there is the possibility of the biochemical formation of hydroxyl radical.

Thus the two main toxic products of oxygen produced during its reduction are O_2^- and H_2O_2, and the entities that may be generated by these active products. Aerobic, facultative and some anaerobic organisms possess enzymes which are capable of scavanging both O_2^- and H_2O_2. Catalases and peroxidases have long been known to consume H_2O_2 and more recently superoxide dismutase (SOD) has been shown to eliminate O_2^- (McCord & Fridovich, 1969). Anaerobes can be considered as those organisms unable to use oxygen as a terminal electron acceptor in the generation of energy usually because they lack the terminal cytochromes that transfer electrons to O_2. They fall into two groups, aerotolerant and aerophobic. Aerotolerant anaerobes do not use O_2 but are not drastically harmed by it and they are able to grow both in the absence or presence of oxygen. Aerophobic anaerobes, more commonly called obligate or strict anaerobes cannot grow in the presence of oxygen. Morris (1975) defined an obligate anaerobe as "an organism which is capable of generating energy and synthesizing its substance without recourse to molecular oxygen and demonstrates a singular degree of adverse sensitivity to oxygen which renders it unable to grow in air at 1 atmosphere". Obligate anaerobes differ markedly in their sensitivity to oxygen (Fredette *et al.*, 1967; Loesche, 1969; Tally *et al.*, 1975; Walden & Hentges, 1975; Rolfe *et al.*, 1977; Rolfe *et al.*, 1978). Loesche (1969) distinguished between two subgroups recognizable among the obligate anaerobes, strict anaerobes which were incapable of growth on plates when the atmosphere contained more than 0,5% oxygen and moderate anaerobes which would grow on plates in the presence of oxygen as high as 2 to 8% and which could be exposed to air for 60 to 90min without appreciable loss of viability. It should be noted however, that many organisms which behave as strict anaerobes when first isolated, become more tolerant after subculture (Willis, 1969).

The difference in oxygen tolerance in different strains of anaerobes shows a general relationship to the effectiveness of the defence mechanisms possessed by the bacteria against the toxic products of oxygen reduction (McCord *et al.*, 1971; Gregory *et al.*, 1978; Hassan & Fridovich, 1979). However, the reasons why obligate anaerobes are intolerant of oxygen is still not completely understood. The action of oxygen in obligate anaerobes appears to occur in two phases (Morris, 1975). During the first phase,exposure to oxygen has a bacteriostatic effect, and the organism appears to be able to protect itself by use of its metabolic reducing power to detoxify oxygen. In many moderate anaerobes there is ample evidence to show that although growth ceases, they are capable of withstanding long exposures to aeration without significant loss in viability. The only effect of oxygen appears to be a cessation of growth and metabolism and recovery is rapid when anaerobic conditions have been restored (Loesche, 1969; O'Brien & Morris, 1971; Onderdonk *et al.*, 1976).

This indicates that many anaerobes are able to sustain viability under aerobic conditions by diverting their reducing power to scavange molecular oxygen at the cost of depressing metabolic function. A similar phenomenon appears to occur in some aerobic bacteria (e.g. nitrogen fixing bacteria) which utilize reducing power to scavange molecular oxygen so as to protect key oxygen labile components from the action of oxygen (Drozd & Postgate, 1970; Jones *et al.*, 1973).

During the second phase, exposure to oxygen becomes bacteriocidal to the cell. The cell appears to be no longer able to overcome the effects of oxygen and irreversible damage leading to cell death results. A number of hypotheses have been proposed to account for the lethal effects of oxygen in anaerobes, and these have been reviewed by Smith & Holdeman (1968), Morris & O'Brien (1971) and Morris (1975,1976). Morris (1976) has summarized the main hypotheses which have been proposed to account for the sensitivity of anaerobes to oxygen as follows:-

(A) Oxygen is Toxic

(1) Presence of free oxygen in the culture medium is incompatible with the attainment and maintenance of low culture Eh value required for growth.
(2) Preferential reduction of exogenous oxygen drains the organism of reducing power which it requires for biosynthetic purposes.
(3) The anaerobe is particularly vulnerable to oxygen because its metabolism and growth depends on key components liable to auto-oxidation.
(4) Growth is inhibited due to oxidation of a key metabolic regulation, presumed either to react directly with oxygen or to be in equilibrium with another auto-oxidizable redox couple.

(B) Oxygen is not itself toxic

(1) Toxic products accumulate in nutrient media exposed to oxygen.
(2) Toxic products (or by-products) are invariably formed by the interaction of oxygen with reduced cell constituents such as iron sulphur proteins, tetrahydropteridines, flavoproteins, as well as the action of certain oxidases and flavin dehydrogenases (hydrogen peroxide, superoxide anion, hydroxyl radical and singlet oxygen are the chief contenders).

Early assumptions that oxygen is toxic by virtue of its excellence as an oxidant have not been borne out by experimental evidence. The fact that many moderate anaerobes are capable of withstanding long exposures to aeration without suffering an apparent damage argues against anaerobes containing unique targets within the cell which are sensitive to oxidation. In addition, the non-specific elevation of Eh in a culture by oxygen is not sufficient to account for the inhibition of growth in anaerobes (O'Brien & Morris, 1971; Onderdonk *et al.*, 1976). Nevertheless, the addition of reducing agents which lower the Eh of the medium, has been shown to facilitate the initiation of growth of anaerobes, especially with small inocula (Morris, 1975).

Recently the emphasis has shifted to the theory that obligate anaerobes contain both fewer and lower levels of the defensive agents known to be active against the toxic products of oxygen reduction. For a number of years it was thought that a lack of the enzymes catalase or peroxidase in anaerobes accounted for their oxygen sensitivity. However, Holdeman & Moore (1972) showed that a large number of obligate anaerobes produced these enzymes. There appear to be a number of reasons why catalase activity was previously undetected in anaerobes. A requirement for exogenously supplied hemin has been shown in *Bacteroides* species (Gregory *et al.*, 1977a). In addition, Gregory *et al.* (1977b) showed that catalase production in a number of *Bacteroides* species was suppressed by the presence of glucose and other carbohydrates. Since anaerobes are routinely cultured in media containing carbohydrates, this would further explain why this enzyme might have escaped detection initially. Wilkins *et al.* (1978) have shown that a number of factors relating mainly to the availability of hemin also affect the production of catalase in *Bacteroides*.

In addition to enzymes which are able to detoxify hydrogen peroxide, all aerobes and facultative organisms possess enzymes which are able to dispose of the superoxide anion harmlessly. McCord

& Fridovich (1969) were the first to discover a group of enzymes which have been termed superoxide dismutases (SOD). *E.coli* was shown to possess 2 such enzymes, an iron containing SOD which was located in the periplasmic space, and a manganese SOD which occurs inside the cell (Gregory *et al.,* 1973). McCord *et al.* (1971) proposed the superoxide theory of obligate anaerobiosis, as a consequence of finding SOD activity lacking in strict anaerobes. They proposed that the lack of this enzyme accounted for the aero intolerance of anaerobes. Later work involving mutants of *E.coli* which lacked peroxidase and catalase activity, and others which lacked SOD in addition, showed an enhanced susceptibility of these bacteria to the lethal effects of oxygen (Hassan & Fridovich, 1979). Although there does appear to be a correlation between the lack of SOD production and oxygen sensitivity, a large number of exceptions have been reported. An aerotolerant anaerobe *Lactobacillus plantarium* lacking both catalase and SOD was reported by Gregory & Fridovich (1974). Some SOD activity was also found in various aero intolerant bacterial species including *Chlorobium, Desulfovibrio* and *Clostridium* (Morris, 1976) and *Bacteroides distasonis* (Gregory *et al.,* 1977a). In a survey of SOD in anaerobes (Gregory *et al.,* 1978) found SOD present in 23 out of 28 of the *Bacteroides* strains tested. In addition several clostridia, anaerobic cocci and anaerobic Gram-positive non-sporing rods contained measurable SOD, although the frequency of SOD occurrence was much larger than in the *Bacteroides*. This data indicated that there was a large variation in SOD levels between genera and among species within a genus of anaerobic bacteria. In several of the *Bacteroides* species the levels of SOD were greater than the enzyme levels reported in *E.coli*. However, these workers did find evidence to suggest that there was a direct correlation between SOD levels and the oxygen sensitivity of a particular strain. Correlations between SOD content and oxygen tolerance in *Bacteroides* was also reported by Carlson *et al.* (1977) and Tally *et al.* (1977), suggesting that SOD in anaerobes may be important in affording some protection to organisms exposed to air for short periods. Although the finding of SOD activity in a number of anaerobes does not disprove the argument of McCord & Fridovich, they do however indicate that further information of the role of SOD in the sensitivity and tolerance of anaerobes is necessary.

Present evidence indicates that there is probably no single cause of oxygen toxicity in anaerobes and that the differences between aerobes and anaerobes appear to be quantitative rather than qualitative.

Although obligate anaerobes are restricted to growing in environments low in redox potential they have very widespread distribution. Anaerobic environments include muds and other sediments of lakes, rivers and oceans; bogs, marshes and many other environments containing decaying organic material; deep underground areas such as in oil pockets and some underground waters; the intestinal tracts of animals; the oral cavity of animals especially around the teeth; certain sewage treatment systems; and canned foods. In most of these habitats the low redox potential is due to the activities of organisms, mainly bacteria, that consume oxygen during respiration. If no replacement oxygen is available the habitat becomes anaerobic. The vast majority of obligate anaerobes are prokaryotes. The only obligate anaerobes found amongst the eukarotes are certain groups of protozoa and it is probable that in most or all of these groups anaerobiosis has been acquired as a secondary characteristic.

Anaerobes which inhabit the gastro-intestinal tract of man and laboratory animals, and the rumen of herbivors have been the subject of intensive study in recent years. The intestinal tract of man is known to be anaerobic (Askevold, 1956; Moore *et al.*, 1969) and the predominant flora (*c.*97%) has been shown to be anaerobic bacteria (Schaedler & Costello, 1963; Drasar, 1967; Finegold, 1969; Moore *et al.*, 1969; Haenel, 1970; Moore & Holdeman, 1972). The number of bacteria increases down the digestive tract (Drasar & Hill, 1974) with few bacteria occurring in the duodenum and jejunum, a more permanent flora in the ileum and larger numbers being present in the large intestine or faeces. The most prevalent micro-organism is *B.fragilis* with an average count of 10^{10} to 10^{11} per g and Moore *et al.* (1969) identified 46% of the 230 strains of faecal bacteria isolated from 2 people as belonging to this species. In addition, 36 other species were isolated from the same specimens. In addition to *Bacteroides* a number of other genera of Gram-negative and Gram-positive anaerobes are common (Dubos *et al.*, 1963; Drasar, 1967;Finegold, 1969; Moore *et al.*, 1969; Finegold, 1970).

The significance of the intestinal flora is still highly speculative. Ruminants are dependent on bacterial action for the utilization of their normal diet (Bryant & Burkey, 1953), but although bacterial products are absorbed by the body from the human colon the extent of their contribution to nutrition is uncertain. An intestinal flora is not essential for life, as generations of germ-free animals have been bred. The normal intestinal flora is also being increasingly implicated in cancer of the large intestine by virtue of their ability to metabolise procarcinogens to produce carcinogens (Drasar & Hill, 1974; Wilkins & Hackman, 1974; Salyers *et al.*, 1977).

In addition to the occurrence of anaerobic bacteria in the intestinal tract, anaerobes have been shown to outnumber aerobes 30:1 on the mucous membrane of the mouth and 10:1 on the surface of the human skin (Rosebury, 1962).

Anaerobes, especially the Gram-negative non-sporing bacilli of the indigenous microflora are recognised as important agents in clinical infections. They have been shown to be implicated in a variety of clinical conditions such as pneumonia, bacteremia appendiceal abscesses, peritonitis, diventriculitis and post-operative infections following abdominal surgery (Dubos *et al.*, 1963; Smith & Holdeman, 1968; Finegold, 1969; Finegold, 1970; Felner & Dowell, 1971; Martin, 1971; Sutter *et al.*, 1975). However, of the more than 300 anaerobic species known to reside in or on human body surfaces, only a few species are consistently isolated from serious infections (Kasper, 1979) and it is likely that virulence factors are important in the selection of these species. Organisms of the genus *Bacteroides* represent the major group of obligate anaerobes involved in human infections, and usually cause either bacteremia or localized abscesses (Kasper *et al.*, 1979). Of the numerous species of *Bacteroides*, *B. fragilis* is the single most frequent clinical isolate. Kasper *et al.* (1979) have also shown that encapsulated strains were able to produce abscesses in experimental animals whereas those lacking capsules were not. Although encapsulated strains of *B.fragilis* represent only about 0,5% of bacterial flora they account for 70-80% of all clinical isolates.

Bacteroides, in particular *B.fragilis*, presents a number of problems in chemotherapy as they are relatively resistant to a number of antibiotics (Finegold & Sutter, 1971; Thornton & Cramer, 1971; Kislak, 1972; Martin *et al.*, 1972; Sutter & Finegold, 1976). In addition an increasing percentage of bacteria isolated from clinical infections are resistant to previously useful antibiotics (Nastro & Finegold, 1972; Murray & Rosenblatt, 1972). This has led to the speculation that antibiotic resistance may be plasmid coded in *Bacteroides*.

Stiffler *et al.* (1974) were the first to report the presence of plasmids in *Bacteroides*. They isolated plasmids from 3 strains of *B.fragilis*, all 3 contained small plasmids with multiple copies per cell, and in addition one strain also contained a large plasmid which was present in only a few copies per cell. This initial report was followed by further reports. Damle & Syed (1975) isolated two plasmids from strains of *B.ochraceus* and one plasmid from a strain of *B. melaninogenicus*, and Guiney & Davis (1975) isolated a single plasmid from *B.fragilis* and two plasmids from a strain of *B.ochraceus*. In a further study by Tinnell & Macrina (1976) plasmids from 2 strains of *B.fragilis* and two strains of *B.thetaiotaomicron* were characterized.

It was initially thought that chromosomal and R plasmid transfer was inhibited in *E.coli* and other enterobacteria under anaerobic conditions (Fisher, 1957; Mitsuhashi, 1965). However, Stallions & Curtiss (1972) showed that chromosome transfer in *E.coli* can occur at high frequency under anaerobic conditions and Moodie & Woods (1973a) reported R plasmid transfer in *E.coli* under anaerobic conditions. Later work by Burman (1975) showed that transfer of certain R plasmids was unaffected by anaerobiosis while the transfer of others was blocked. Initial attempts to obtain transfer of antibiotic resistance between strains of *E.coli* and *Bacteroides* were unsuccessful (Anderson & Sykes, 1973; Guiney & Davis, 1975; Del Bene *et al.*, 1976). Burt and Woods (1976) did succeed in obtaining transfer of an R plasmid from *E.coli* to *Bacteroides* after heat treatment of the recipient which possibly inhibited the restriction system of the anaerobe. A report of a successful multiple antibiotic transfer from *B.fragilis* to *E.coli* was published by Mancini & Behme (1977), however, the individual resistance markers were unstable in the transconjugant and the presence of closed circular plasmid DNA was not demonstrated in either the *B.fragilis* donor or the *E.coli* recipient. Guiney & Davis (1978) were the first to positively identify an R factor harboured by a *Bacteroides* strain. The pG10 plasmid of *B.ochraceus* has been identified as a conjugative plasmid carrying the genes to tetracycline, chloramphenicol kanamycin and streptomycin. They showed that this plasmid could be transferred to *E.coli* by conjugation and is stably maintained in the recipient. Recently 4 reports of plasmid mediated transferable resistance between *Bacteroides* strains have appeared in the literature. Tally *et al.* (1979) reported the transfer of clindamycin and erythromycin resistance between a strain of *B.fragilis* and *B.thetaiotaomicron*. Privitera *et al.* (1979 a) reported the transfer of multiple antibiotic resistance (clindamycin, erythromycin and streptogramins) between strains of *B.distasonis* and *B.fragilis* and Welch *et al.*(1979) reported transferable lincosamide-macrolide resistance in *Bacteroides*. A fourth report by Privitera *et al.*(1979 b) described the transfer of tetracycline resistance between *B.fragilis* species which was induced by growing the donor bacterium in tetracycline before mating. Their results suggest that tetracycline resistance is borne on a transferable plasmid whose conjugative transfer requires activation of the tetracycline resistance genes and that a common regulatory mechanism involving both expression and conjugative ability may be involved.

The finding of R plasmids which have conjugative activity in *Bacteroides* has a number of important implications. The presence of R plasmids can only act to compound the problems which have already been encountered in chemotherapy. In addition, obligate anaerobes far outnumber facultative anaerobes in the colon, and such large populations may provide scope for the evolution of new R plasmids. As transfer between *Bacteroides* and *E.coli* has been shown to occur (Guiney & Davis, 1978), *Bacteroides* could provide a pool of resistance plasmids capable of infecting facultative pathogenes such as *Salmonella* and *Shigella.*

Although plasmid transfer has been demonstrated in bacteroides, the transfer of chromosomal genes has not been observed. The need for genetic studies on obligate anaerobes, particularly in the areas of anaerobic physiology (Morris, 1975) and the causes of virulence (Bartlett & Onderdonk, 1979) appears to be essential for future progress. However, the difficulties of working with obligate anaerobes seem to have been responsible for failure to establish a system for genetic analysis and manipulation in any of these organisms. Amongst the Gram-positive anaerobes, *Clostridium perfingens* appears to be a good candidate for such studies (Sebald & Costilow, 1975). Amongst the Gram-negative anaerobes, *B.fragilis* has been suggested as an appropriate choice due to its medical importance (Van Tassell & Wilkins, 1978). In addition, it has a number of characteristics which make it a suitable choice for such experiments. For example, it can tolerate prolonged exposure to air, it is possible to culture it on both complex and minimal media and it is relatively easy to isolate phages which infect these organisms (Van Tassell & Wilkins, 1978; Booth *et al.*, 1979). With this end in view a study of some aspects of the genetics of several members of the "*Bacteroides fragilis* group" was undertaken. The results obtained from the initial investigations indicated that further studies on some of the molecular aspects pertaining to mutation, recombination and repair might prove informative. The thesis has thus been divided into 2 parts. Part I dealing with a study of some aspects of the genetics of the *B.fragilis* group and part II dealing with the physiological responses of *B.fragilis* to far UV radiation.

PART I

GENETIC STUDIES ON THE *BACTEROIDES FRAGILIS* GROUP

CHAPTER II

THE ISOLATION OF MUTANTS IN THE *BACTEROIDES FRAGILIS* GROUP

2.1 INTRODUCTION

Although *Bacillus fragilis* was first described by Veillon & Zuber in 1898 it was not until the work of Holdeman & Moore (1974) that the taxonomy of this organism was placed on a sound footing. They examined 326 stains of bacteria with characteristics which fitted earlier descriptions of *B.fragilis*. They found clusters of strains within the group which had phenotypic characteristics that were similar to those reported by Eggerth & Ganon (1933) for *B.fragilis*, *B.thetaiotaomicron*, *B.distasonis*, *B.ovatus* and *B.vulgatus*. However, since the strains were morphologically and phenotypically quite similar and had similar guanine : cytosine ratios they regarded them as all belonging to subspecies of *B.fragilis*. Later DNA homology studies by Cato & Johnson (1976) have shown that these subgroups are genetically distinct and for this reason the former subspecies have been reinstated to species rank. These species include *B.fragilis*, *B.thetaiotaomicron*, *B.ovatus*, *B.distasonis* and *B.vulgatus*. Two previously recognized species, *B.eggerthii* and *B.uniformis* homology group 3452-A and *B.fragilis* subspecies "a" are also included in this group (Johnson, 1978; Johnson & Ault, 1978). However, these former subspecies are still often referred to as the "*Bacteroides fragilis* Group" (Booth *et al.*, 1979).

In an early study on the growth requirements of *B.fragilis*, Tamimi *et al.* (1960) reported that it failed to grow in a chemically defined medium. Later work by Quinto (1962, 1966) and Quinto & Sebald (1964) showed that these organisms had simple nutrient requirements and needed only glucose, minerals, hemin, bicarbonate and carbon dioxide for growth. In a further study on the nutritional features of *B.fragilis* Varel & Bryant (1974) found that the essential component which was absent from the media of Tamimi *et al.* (1960) was hemin. They found that casitone provided sufficient hemin for growth, but when this was replaced by cysteine, no growth occurred. These workers developed a minimal medium which supported the growth of *B.fragilis*. Similar growth requirements were found for most other members of the genus *Bacteroides* (Caldwell & Arcand, 1974). Lev *et al.*(1971) reported that the hemin requirements of *B.melaninogenicus* could be replaced by succinate and vitamin K. This strain has been shown to form cytochrome b from the added heme (White *et al.*, 1962). However, strains grown on succinate differed from those grown on hemin, as they were not inhibited by cyanide (Lev *et al.*, 1971).

Varel & Bryant (1974) also found that *B.fragilis* had a very limited ability to utilize organic nitrogen compounds such as amino-acids as the sole source of nitrogen and NH_4^+ is the preferred nitrogen source. Miles *et al.* (1976) reported that amino acids actually inhibit the growth of *B.melaninogenicus*, although this strain can utilize amino acids when present as peptides. Burt (1977) working with *B.thetaiotaomicron*, and Mossie *et al.* (1979) working with *B.fragilis* both reported that these strains were unable to incorporate labelled amino acids. Varel & Bryant (1974) suggested that the great biosynthetic ability of these organisms is related to their ecological niche within the large intestine where many nutrients such as amino acids are in very low supply, whereas materials such as ammonia, heme and vitamin B12 must be available much of the time. This suggests that many

Bacteroides strains may be more permeable to peptides than to amino acids, as there is little survival value in the maintenance of systems (presumably transport systems) for the utilization of preformed cell monomers such as amino acids. However, it does not appear to be a universal feature of the group as Van Tassell & Wilkins (1978) succeeded in isolating several amino acid auxotrophic mutants from two strains of *B.fragilis*. Arginine has also been reported to be a growth limiting factor in another anaerobe, *Eubacterium lentum*, where it appeared to be utilized as a sole energy source (Sperry & Wilkins, 1976).

The isolation of stable mutants is an essential step in any genetic study investigating the transfer of chromosomal markers. The literature contains very few reports of the isolation of such mutants from obligate anaerobes. Three mutant strains of *Coprococcus* species were isolated by Thompson *et al.* (1976) which could not utilise phloroglucinol as a carbon source. Sebald & Costilow (1975) isolated a group of specific auxotrophic mutants in *Clostridium perfringens* which were used to examine the potential for exchange of chromosomal markers. Several auxotrophic mutants including both amino acid mutants and nucleotide mutants have been isolated from two strains of *B.fragilis* by Van Tassell & Wilkins (1978) using ethane methane sulphonate (EMS) as a mutagenic agent. They utilized a semi-selective penicillin enrichment technique to obtain these mutants, with the incorporation of clavulanic acid to increase the susceptibility of the *B.fragilis* strains to penicillin (Wüst & Wilkins, 1978).

Although the report by Adelberg *et al.* (1965) on the optimum conditions for the uses of the potent mutagen N-methyl-N$^-$-nitro-N-nitrosoguanidine (NTG) for the induction of mutants in *E.coli* is regarded as definitive by many workers, these methods are not suitable for all bacteria. Hopwood (1970) has pointed out that different organisms rarely respond in precisely the same way to a mutagenic treatment or a mutant screening program. Consequently a number of different mutagenic treatments and selection techniques were attempted in order to obtain stable chromosomal mutants of *B.fragilis*.

2.2 MATERIALS AND METHODS

2.2.1 BACTERIAL STRAINS

The characteristics of the 36 strains of *Bacteroides* used in this study are listed in Appendix I. The majority of the strains were obtained from the Department of Microbiology in the Faculty of Medicine at the University of Natal, Durban. The strains were isolated from clinical infections and identified in the Durban Department. Two strains were isolated in the Department of Microbiology at Rhodes University from faecal material, and one strain was donated by Dr. L.V. Holdeman, V.P.I. Anaerobe Laboratory, Blacksburg, U.S.A.

The strains were maintained on brain heart infusion (BHl) agar slopes and beef liver anaerobe media and were subcultured at 6 monthly intervals. Attempts to store the cultures by freeze drying did not prove to be very successful. In addition all 36 strains were maintained on agar plates which were subcultured at 4 weekly intervals over a two year period in order to monitor variations in the colonial morphology of the strains. Strains being used in experiments were maintained on stock plates which were subcultured at weekly intervals.

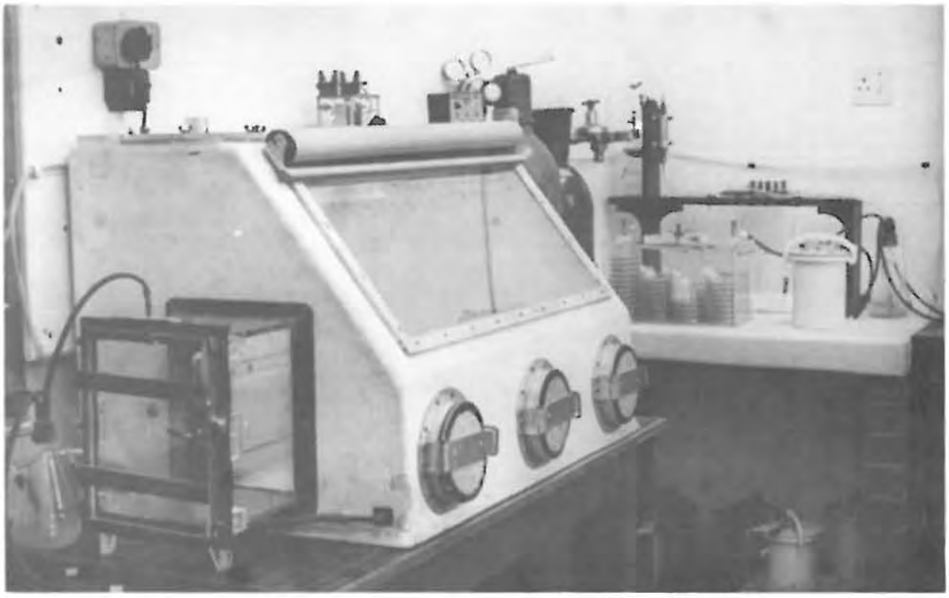
2.2.2 ANAEROBIC TECHNIQUES

A mixture of *c*.95% carbon dioxide (CO_2) and 5% hydrogen (H_2) was used to provide an anaerobic atmosphere in the anaerobic glove box, anaerobic containers, and for gassing culture media. A mixture of commercial grade CO_2 and H_2 was passed through a deoxycatylist (Engelhard) and into a distribution manifold. The flow rate of the gasses was measured by means of rotameters (Air Products) and the proportion of gasses was controlled manually. The distribution system used for flushing anaerobic containers with the CO_2 + H_2 mixtures, and the gassing apparatus used to prepare pre-reduced anaerobically sterilised media (PRAS Media) are shown in Plate 2.1 A and B.

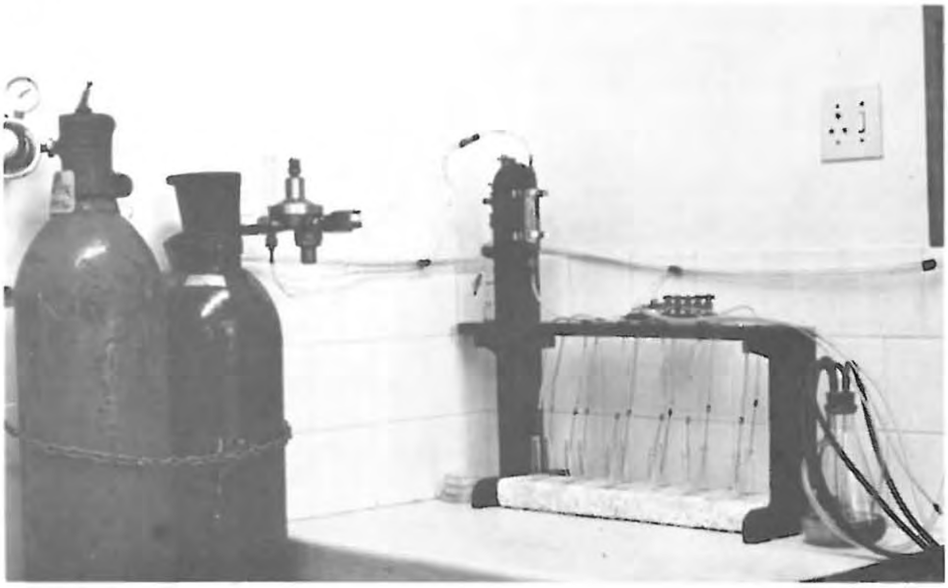
PRAS media was prepared according to the methods of Moore (1966) and Hungate (1969) (See Appendix II). Anaerobic media contained reducing agents, and were stored under anaerobic conditions until required. Hungate tubes were used for liquid media and Astell roll tubes were used for agar slopes. Commercially available anaerobic jars (Gas Pack, and Baird & Tatlock) or large perspex containers which could hold 90 agar plates, were used for anaerobic storage and incubation of agar plates (Plate 2.1 A and C). As the perspex containers were not pressure vessels, anaerobic conditions were established by flushing with CO_2 and H_2 for 30 to 45 min. Approximately 5g of palladium catalyst and 200g silica gel (500 g for drying plates) were placed in the containers before perfusing. The gas-tight lids of the containers were fitted with O ring seals which allowed anaerobic conditions to be maintained in the containers for up to 4 weeks.

The anaerobic glove cabinet used in this study has been described by Moodie and Woods (1973b) and it incorporated various features of the cabinets constructed by Aranki *et al.* (1969) and Leach *et al.* (1971)

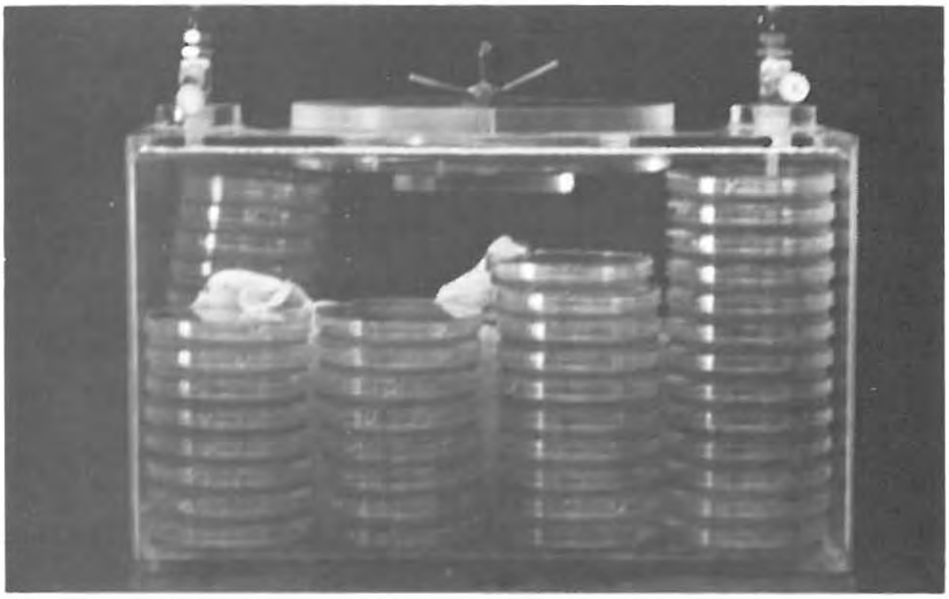
PLATE 2.1



(A) ANAEROBIC GLOVE BOX



(B) ANAEROBIC GASSING APPARATUS



(C) ANAEROBIC INCUBATION CONTAINER

(Plate 2.1 A). The cabinet was flushed with the $CO_2 + H_2$ gas mixture before and during use and the interior of the cabinet was kept under a slight positive pressure. In addition 0,5% palladium coated alumina pellets (*c.* 100g) were placed in a perforated metal tray in the cabinet to remove any traces of oxygen. If the cabinet had not been in use, gassing for 4 to 6 h was required to establish anaerobic conditions using a flow rate of 4-6 $1\ min^{-1}$. For routine use the cabinet was flushed for 3 h before use. The entry port on the side of the cabinet required flushing for 20 min. before materials could be transferred into the cabinet.

As the *Bacteroides* strains used in this investigation could all tolerate short periods of exposure to air without suffering any loss of viability, many experimental manipulations were carried out on the laboratory bench. Inoculations into broth were either made by injection through the top of a Hungate tube or by inoculation with a platinum loop followed by perfusion of the tube with CO_2 and H_2. In a number of experiments however, all manipulations and procedures were carried out in the anaerobic glove cabinet so that at no time were cells exposed to oxygen during the experiment.

2.2.3 THE GROWTH CHARACTERISTICS OF *B.FRAGILIS*

The growth rates of 4 of the *B.fragilis* strains, (PS1, PS2, PB1, P1), in BH1 and liquid minimal medium (MM), were determined by measuring turbidity at 540 nm and calibration curves for each strain grown under a particular set of conditions were determined (Fig. 2.1). Calibration curves for the colorimetric determination of the concentration of *B.fragilis* cells in broth (number of c.f.u. ml^{-1}) were made using the results obtained from plate viability counts (Fig. 2.2). The growth of *B.fragilis* cells in different variations of MM was determined.

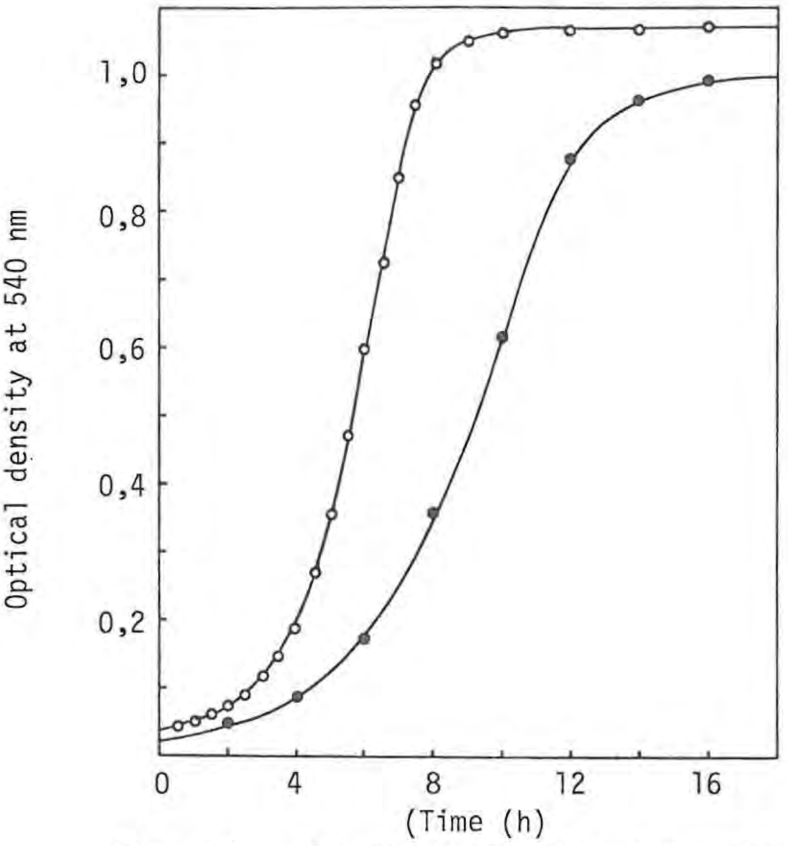


Fig. 2.1. Growth of wild type *B.fragilis* (PS1) in BHI broth (o) and MM (●).

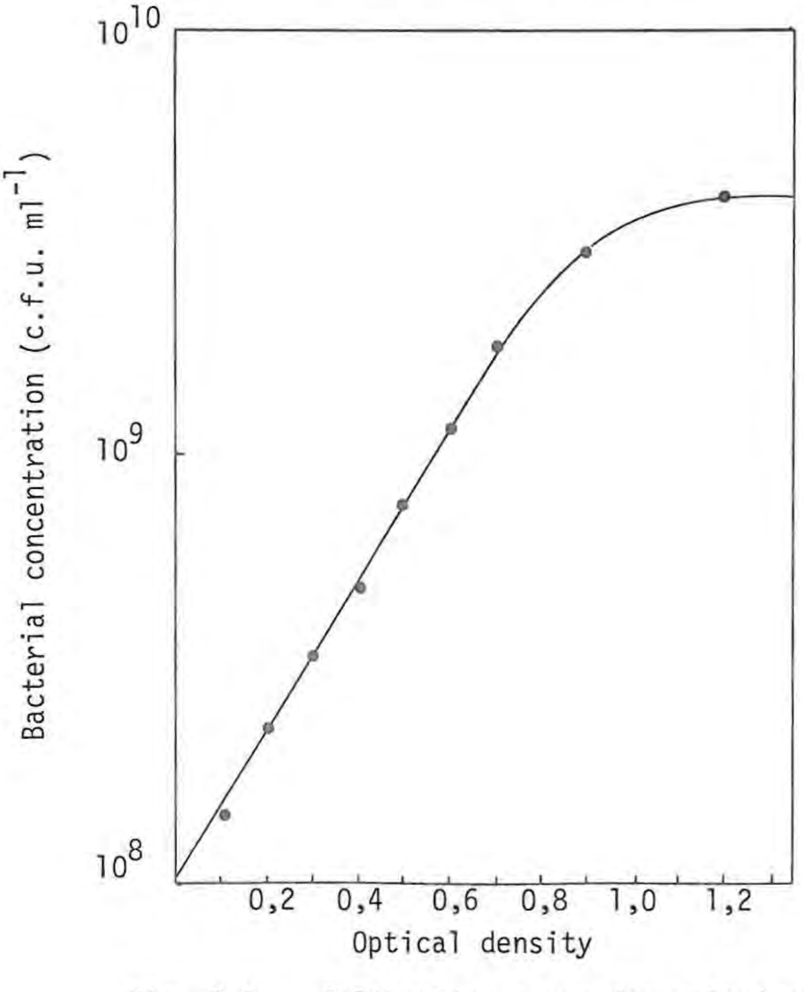


Fig. 2.2. Calibration curve for colorimetric determination of the concentration of *B.fragilis* (PS1) cells in BHI broth culture.

2.2.4 VIABILITY OF *B.FRAGILIS* CELLS UNDER AEROBIC AND ANAEROBIC CONDITIONS

The viability of *B.fragilis* cells plated under anaerobic conditions in the glove cabinet, and under aerobic conditions on the laboratory bench was determined. The viability of *B.fragilis* cells held for 24 h in a variety of holding solutions under non-growing conditions was determined, both for aerobically and anaerobically held cells, by means of plate viability counts.

2.2.5 ANTIBIOTIC AND INHIBITOR SENSITIVITY TESTS

The antibiotic susceptibility patterns of a number of strains of *B.fragilis* were determined by the sensitivity disc method (Sapico *et al.,* 1972). Minimal inhibitory concentrations (m.i.c.) values for different antibiotics, were determined either by inoculating fixed volumes of cells into BHl broth containing doubling dilutions of the antibiotic or inhibitor, or by streaking broth cultures onto BHl agar plates containing doubling dilutions of the antibiotic or inhibitor. The m.i.c. was defined as the lowest concentration which completely inhibited growth after incubation for 24 h.

2.2.6 CELL SURVIVAL AFTER TREATMENT WITH MUTAGENIC AGENTS

B.fragilis cells were grown in BHl broth until mid exponential phase (OD = 0,3 or 0,5), and in some cases stationary phase cells (15 h) were also used. The cells were centrifuged and resuspended in BHl broth, 0,1 M-Tris buffer (pH 7,6) or Ringer solution. Cells were then given treatments with a variety of mutagenic agents and samples were taken at fixed time intervals and the surviving fraction of cells determined by means of plate viability counts.

(i) NTG Treatment

NTG was added to exponential phase cells suspended in 10 ml BHI broth or 0,1 M tris-maleate buffer pH 6,0 or 9,0 to final concentrations of 100, 300 and 1 000 μg ml^{-1}. Cultures were incubated at 37°C and samples were removed at fixed time intervals, centrifuged and washed to remove the NTG.

(ii) Ethyl Methane Sulfonate (EMS) Treatment

EMS was added to either exponential phase cells or stationary phase cells in BHI broth or suspended in buffer, to final concentrations of 0,5; 1,0; 2,0 and 2,5%. Cultures were incubated at 37°C and samples were removed at fixed time intervals, and plated to determine the surviving fraction of cells.

(iii) Nitrous Acid Treatment

Sodium nitrate ($NaNO_2$) (0,05 M) and acetate buffer, pH 4,5, (3 ml of each) were added to 5 ml exponential phase cells (OD 0,3) in BHI broth. Cultures were incubated at 37°C and samples were removed at fixed time intervals, diluted in broth and plated to determine the surviving fraction of cells.

(iv) ICR 191 Treatment

BHI broth (0,1 ml), 0,1 ml cells and 100 μg ICR 191 were added to 0,8 ml MM and the cultures were incubated at 37°C. Samples were removed at fixed time intervals, diluted in broth and plated to determine the surviving fraction of cells.

(v) Ultra Violet Radiation

Cells in Ringer solution were diluted to 1 x 10^7 cells ml^{-1} and were irradiated with increasing fluences of UV radiation. Samples of the cell suspension were removed at fixed times and plated to determine the surviving fraction of cells.

2.2.7 INDUCTION OF MUTANTS IN *B.FRAGILIS*

Four strains of *B.fragilis* (PS1, PS2, PB1, P1) were treated with NTG, EMS and UV radiation as described in the previous section. In addition the method described by VanTassell & Wilkins (1978) for EMS treatment was also used. After treatment with the mutagens, bacteria were diluted and plated on BH1 agar to determine the number of viable cells. The proportion of auxotrophs was determined by replicating from BH1 agar plates onto MM plates (Lederberg & Lederberg, 1952). Non-replicating colonies were checked by streaking on MM plates. Antibiotic resistant mutants were selected by plating high concentrations of cells onto gradient plates containing rifampicin, chloramphenicol, tetracycline, erythromycin, vancomycin, nitrofurantoin, carbenicillin, cephalosporin and metronidazole (See Appendix II). In addition, plates containing a fixed concentration of antibiotic were also used and the proportion of antibiotic resistant mutants was determined. Temperature-sensitive mutants were selected by incubating replicates on BH1 agar at 37° and 42°C respectively. The techniques for screening and selection of the other mutants (colonial morphology variants, phage resistant mutants, bacteriocin mutants and UV sensitive mutants) are described in the appropriate chapters.

2.2.8 ENRICHMENT PROCEDURES

After the cultures had been incubated in BHI broth to allow phenotypic expression, they were washed three times in Ringer solution, resuspended in MM broth and incubated at 37°C for 2 h to deplete any auxotrophic mutants of the internal nutrients. Penicillin (1 000 μg ml^{-1}) or carpenicillin (100 or 200 μg ml^{-1}) or ampicillin (500 μg ml^{-1}) final concentration, were added to the cells and the change in OD resulting from lysis was monitored. When lysis had occurred the cells were washed 3 times in saline, diluted and plated on BHI agar. In some experiments, the enrichment technique of Molholt (1967) as described by Hopwood (1970) was used which involves two cycles of enrichment. The recently published enrichment technique using clavulanic acid,of Van Tassell & Wilkins (1978) was also used and this method was modified for attempts to isolate UV sensitive mutants (See 10.2.3).

2.2.9 CHARACTERIZATION OF MUTANTS

Auxotrophic requirements were determined by the pool-plate method of Holliday (1956). Back mutations of auxotrophic mutants were determined after further mutagenic treatment.

2.3 RESULTS

2.3.1 GROWTH CHARACTERISTICS OF *B.FRAGILIS*

All the wild type *B.fragilis* strains tested grew relatively well on the MM of Varel & Bryant (1974). The modified minimal media described by Van Tassell & Wilkins (1978) gave somewhat better growth with some of the strains. The addition of casamino acids stimulated growth whereas the addition of amino acid mixtures had little effect on growth and in some strains a slight inhibition in growth occurred.

2.3.2 THE VIABILITY OF CELLS PLATED AND HELD UNDER AEROBIC AND ANAEROBIC CONDITIONS

No loss in viability occurred when cells were plated on the laboratory bench during which time they were exposed to the air for *c.* 30 min. *B.fragilis* cells held in various holding solutions under non-growing conditions for 24 hours showed a loss in viability to varying degrees. In cells held under aerobic conditions BHI solution and Ringer solution (RS) both showed quite good holding properties, with between 20 and 50% of the cells still being viable after 24 h (Fig. 2.3). Even when cells were continuously aerated by shaking for 24 h 10% of the cells were still viable. In cells held under anaerobic conditions both the anaerobe solution (AS) and the minimal salt solution (MS) had good holding properties with over 80% of the cells still being viable after 24 h (Fig. 2.4). No holding solution was found which had good holding properties under both anaerobic and aerobic conditions.

2.3.3 ANTIBIOTIC SUSCEPTIBILITY TESTS

The 4 strains of *B.fragilis* tested for susceptibility to various types of antibiotics all show fairly similar sensitivity patterns (Table 2.1). The m.i.c. values for one of the strains (PS1) were determined (Table 2.2).

2.3.4 INACTIVATION AND MUTAGENESIS BY MUTAGENIC AGENTS

Treatment with NTG (100, 300 and 1 000 $\mu g\ ml^{-1}$) resulted in a progressively rapid loss of viability in *B.fragilis* cells (Fig. 2.5). Sensitivity to NTG was much greater at pH 9 than at pH 6. EMS

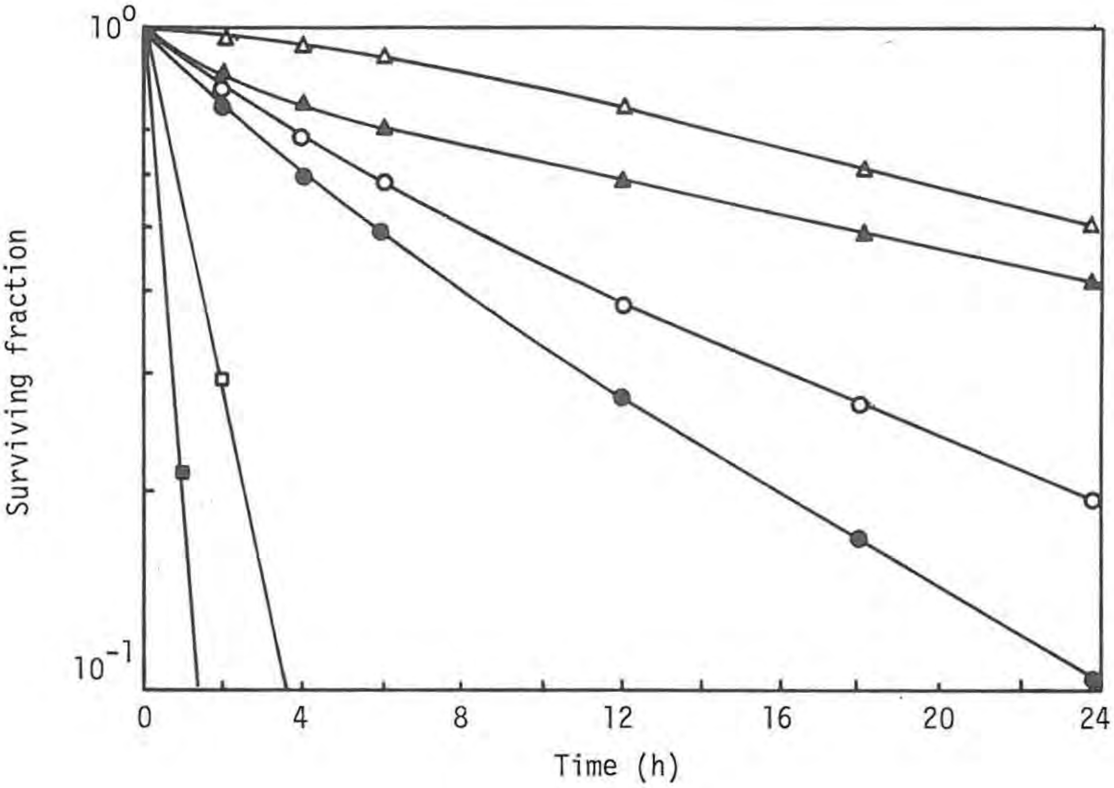


Fig. 2.3. The viability of *B.fragilis* PS1 cells held under non-growing conditions in aerobic holding solutions. Cells held in BHI holding solution (△), RS + peptone (▲), RS (o), RS shaken (●), AS (□), and MS (■).

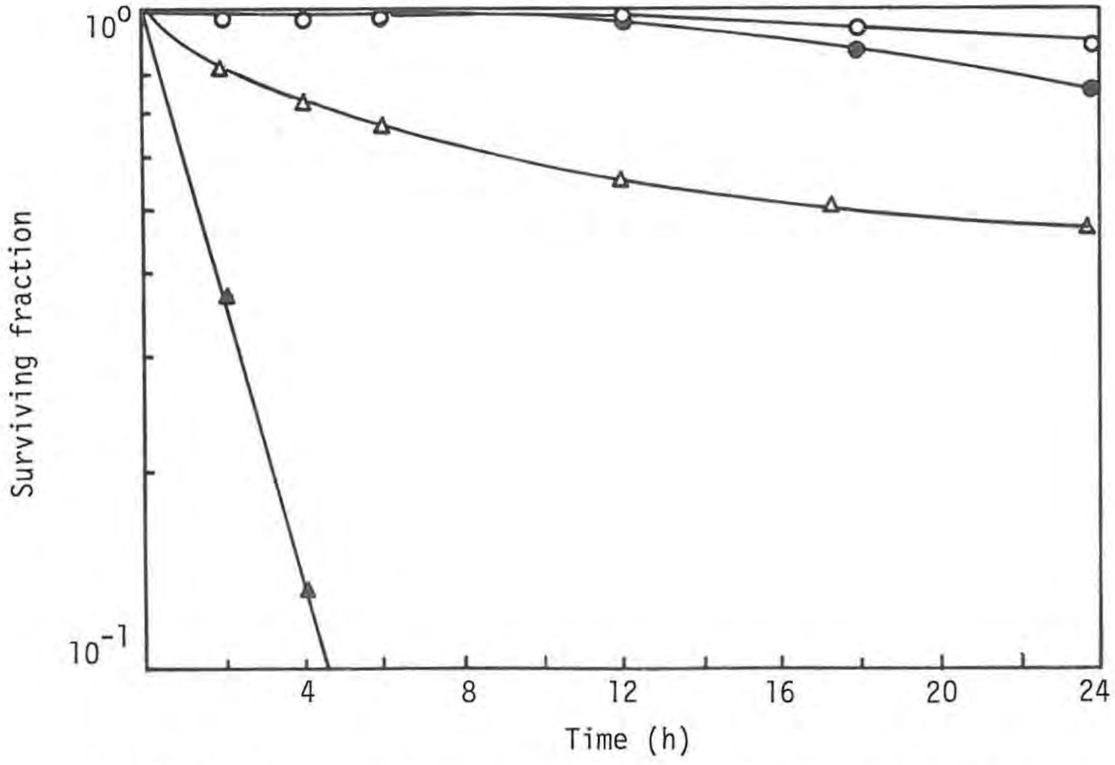


Fig. 2.4. The viability of *B.fragilis* PS1 cells held under non-growing conditions in anaerobic holding solutions. Cells held in AS (o), MS (●), MS + peptone (△) and RS (▲).

TABLE 2.1

Antibiotic sensitivity of *B.fragilis* strains.

		B.fragilis strains			
Antibiotic	Concentration	PS1	PS2	PB1	P1
Penicillin G	1,5 units	R	R	R	R
Ampicillin	25 μg	R	S	R	R
Carbenicillin	50 μg	R	R	R	S/R
Methacillin	25 μg	R	R	R	R
Cloxacillin	5 μg	R	R	R	R
Cephalosporin	30 μg	R	R	R	R
Chloramphenicol	10 μg	S	S	S	S
Streptomycin	10 μg	R	R	R	R
Tetracycline	30 μg	S	S	S	S
Erythromycin	25 μg	S	S	S	S
Rifampicin	25 μg	S	S	S	S
Colistin	200 μg	R	R	R	R
Kanamycin	30 μg	R	R	R	R
Vancomycin	20 μg	S	R	R	R
Clindamycin	10 μg	S	S	S	S
Gentamycin	10 μg	R	R	R	R
Nalidixic acid	30 μg	R	R	R	R
Nitrofurantoin	15 μg	S	S	S	S
Fusidic acid	30 μg	S	S	S	S
Metronidazole	5 μg	S	S	S	S
Tobramycin	10 μg	R	R	R	R
Mynocycline	30 μg	S	S	S	S
Novobiocin	30 μg	S	S	S	S
Septrin	25 μg	S/R	R	S	S
Sulphonamide	300 μg	R	R	R	R

S = Sensitive R = Resistant S/R = Partial sensitivity

TABLE 2.2

Minimum inhibitory concentration of antibiotics and inhibitors in *B.fragilis* strain PS1.

Antibiotic	m.i.c. $\mu g\ ml^{-1}$
Penicillin G	250
Penicillin G + 10 μg clavulanic acid	32
Carbenicillin	125
Cephalosporin	500
Chloramphenicol	1
Naladixic acid	600
Rifampicin	3
Tetracycline	2
Nitrofurantoin	10
Vancomycin	2
Erythromycin	4
Inhibitors	μg
Caffeine	25 000
Acraflavin	10
Sodium arsenite	30

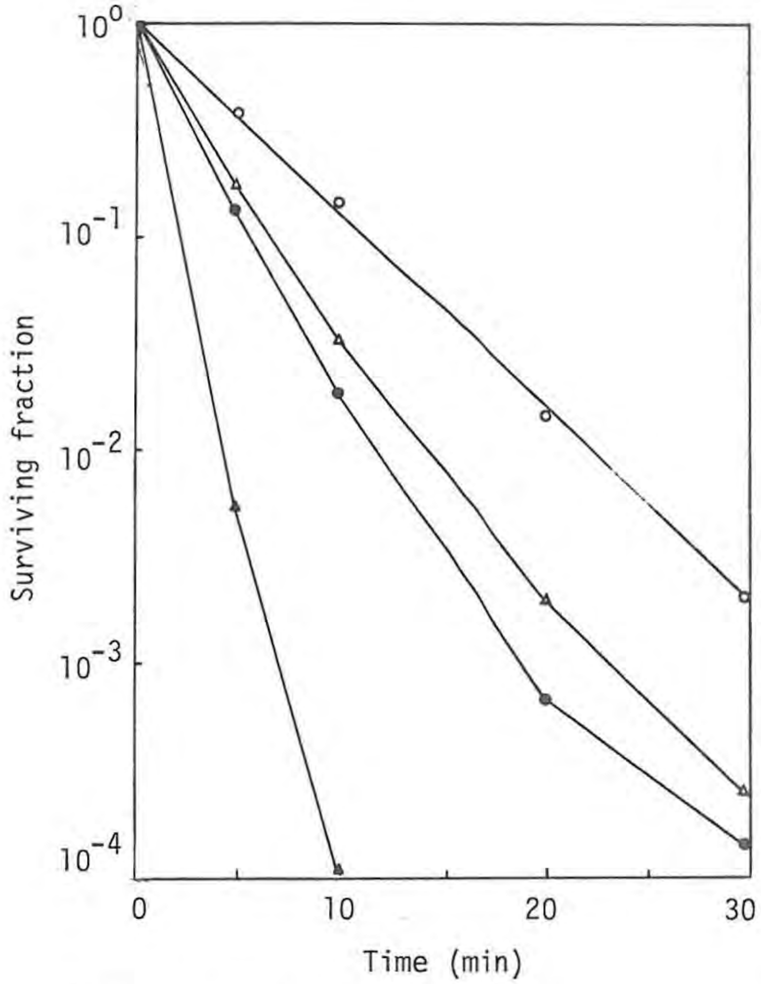


Fig. 2.5. Inactivation of *B.fragilis* (PS1) by NTG at 100 μg ml^{-1} pH 6 (o), 300 μg ml^{-1} pH 6 (△), 100 μg ml^{-1} pH 9 (●), 1 000 μg ml^{-1} pH 6 (▲).

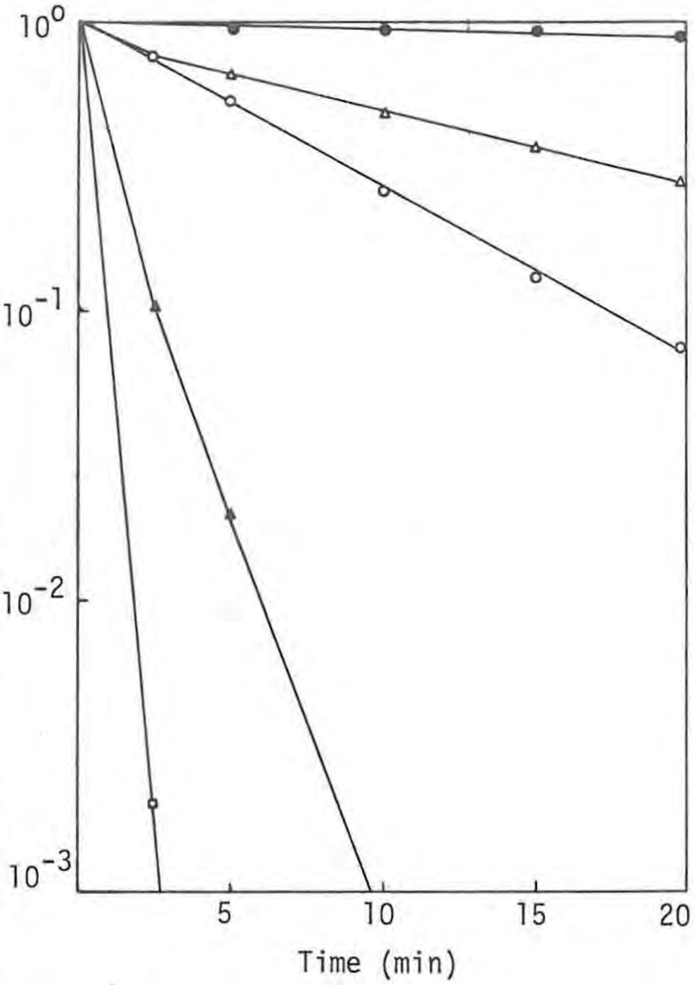


Fig. 2.6. Inactivation of *B.fragilis* (PS1) cells in RS by EMS in exponential phase cells at 0,5 (●), 1,0 (o), 2,0 (▲) and 2,5% v/v (□), and in stationary phase cells at 1,0% v/v (△).

treatment in buffer held cells under aerobic conditions resulted in a much greater loss of viability than cells treated in BH1 broth under anaerobic conditions. In buffer held cells,90% of the cells were still viable after 20 min exposure to 0,5% EMS (v/v), whereas less than 0,01% of the cells were viable after 3 min when cells were treated with 2,5% EMS (v/v) (Fig. 2.6). Stationary phase cells were found to be less sensitive to EMS treatment than exponential phase cells. When 2,5% EMS was added directly to cells in BH1 broth there was *c*.10% survival after 20 min. Exposure to nitrous acid proved very toxic with less than 0,01% of cells still remaining viable after 30 min (Fig. 2.7). With ICR 191 treatment, *c*.2,5% of the cells were still viable after 60 min (Fig. 2.7). Exposure to UV radiation caused a rapid loss in viability of *B.fragilis* cells, stationary phase cells were less sensitive than exponential phase cells (Fig. 2.8).

No auxotrophic mutants were obtained after treatment with nitrous acid, ICR 191, NTG at pH 9, or UV radiation. A small number of auxotrophic mutants was obtained after treatment with NTG at pH 6 and EMS ($<$ 0,1%). In addition a number of antibiotic resistant mutants was also isolated using these treatments (Table 2.3).

2.3.5 ENRICHMENT OF MUTANTS

The enrichment techniques used initially produced very little increase in the number of auxotrophic mutants, in spite of numerous variations tried. The one experiment attempted later using the method described by Van Tassell & Wilkins (1978) indicated that this method was superior to the methods used in the early part of this study.

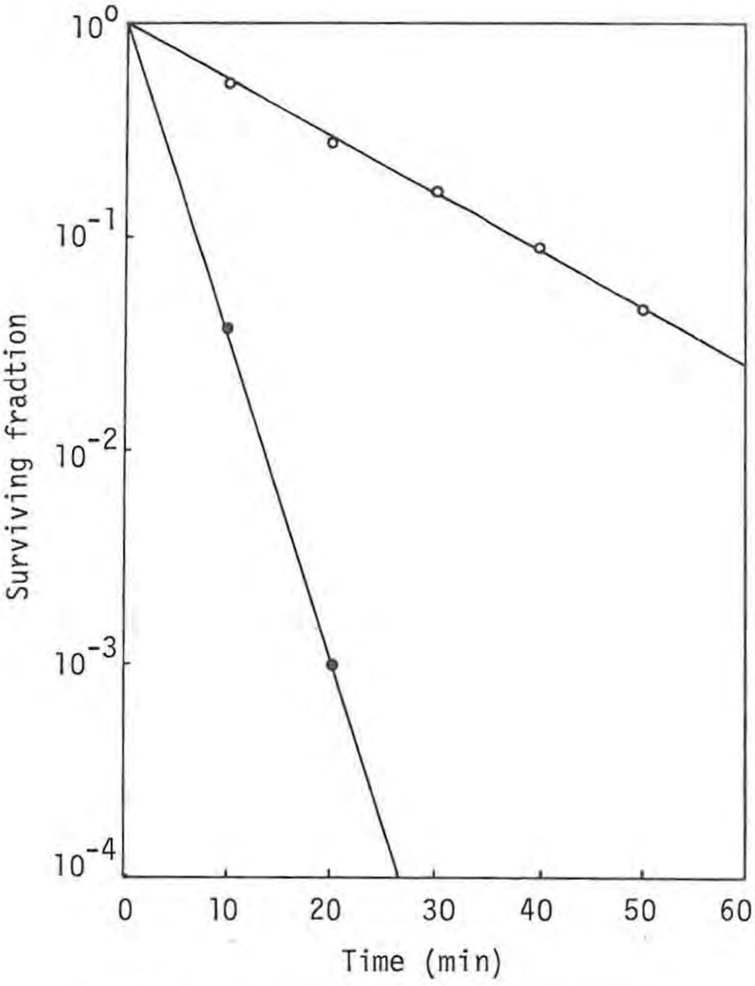


Fig. 2.7 Inactivation of *B.fragilis* (PS1) by ICR191 100 μg ml^{-1} (o), and nitrous acid (●).

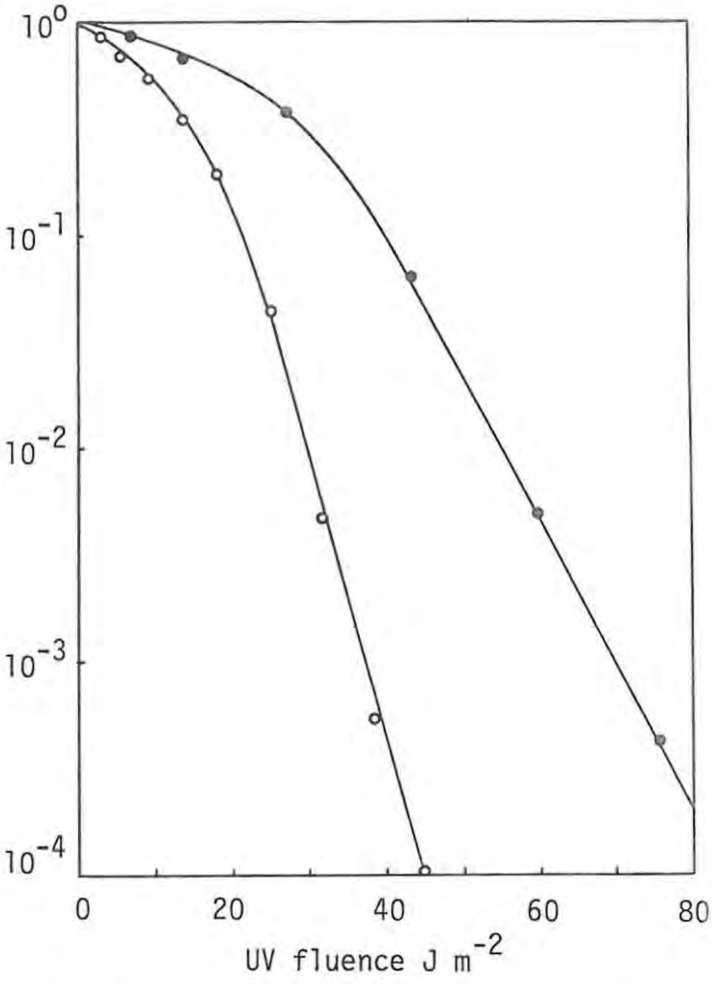


Fig. 2.8. Inactivation of *B.fragilis* cells in RS with increasing fluences of UV radiation in exponential (o) and stationary (●) phase cells.

TABLE 2.3

Mutants isolated from two strains of *B.fragilis*.

Type of mutant		*B.fragilis* strains PS1	PS2
Auxotrophic	Peptide mutants	46	31
	Vitamin mutants	2	0
Antibiotic resistant mutants	Rifampicin	42	-
	Erythromycin	14	-
	Vancomycin	15	-
	Nitrofurantoin	4	-
	Chloramphenicol	0	-
	Tetracycline	0	-
	Carbenicillin	1	-
Temperature sensitive mutants		0	-

2.3.6 CHARACTERIZATION OF MUTANTS

A total of 79 auxotrophic mutants (mutants which would grow on complete media but not on MM) were isolated from 2 strains of *B.fragilis*. Of these 77 of the mutants were found to grow on MM supplemented with peptone of various types but all grew very poorly or not at all on MM supplemented with casamino acids or pooled amino acids. In spite of numerous attempts, no individual amino acid mutants could be isolated from either of these two strains. Two of the auxotrophic mutants grew on MM supplemented with pooled vitamins. One of these mutants was a nicotinimide mutant, the other would not grow on any media supplemented with individual vitamin solutions. Using the nicotinimide mutant no back mutation was observed after further mutagenic treatment.

2.4 DISCUSSION

B.fragilis has been described as a moderate anaerobe by virtue of its ability to withstand exposure to room atmosphere for periods of at least 60 min without loss of viability (Loesche, 1969). The results of the experiments in which cells were held in buffers under aerobic conditions showed that the effect of oxygen was mainly bacteriostatic. The bacteriostatic effect of oxygen on growing *B.fragilis* cells has been reported by Onderdonk *et al.* (1976). The various holding solutions tested showed a marked difference in their holding properties under both anaerobic and aerobic conditions. The reasons for this difference in holding ability is not known.

The scarcity of reports on the isolation of mutants from anaerobic bacteria might suggest that a certain amount of difficulty has been encountered in attempts to isolate mutants from these organisms using standard techniques. In attempting to isolate mutants from *B.thetaiotaomicron*, Burt (1977) experienced considerable difficulty. Van Tassell & Wilkins (1978) also mention that a number of mutagens were tried before a successful method was obtained for the isolation of auxotrophic mutants in *B.fragilis*. In this study two features of the mutagenic treatments tried, have emerged. One has been the very low yield of all types of mutants which were obtained. The second feature was the marked sensitivity of *B.fragilis* to the mutagenic agents. Compared with the mutation yield of well known aerobic bacteria such as *E.coli*, where Adelberg *et al.* (1965) were able to obtain 42,5% auxotrophs after treatment with 1 000 μg ml^{-1} NTG, the yields obtained for *B.fragilis* cells subjected to a similar treatment were of the order of *c*. 0,1% with a maximum of about 0,5%. To some extent these low yields can probably be attributed to the relatively ineffective enrichment techniques used. However, this difficulty has been largely overcome by the penicillin - clavulanic acid enrichment technique of Van Tassell & Wilkins (1978). In addition to auxotrophic mutants, the yields of antibiotic resistant mutants were also found to be low, and were of the order of 1 in 10^{8} cells or less. The results obtained from the inactivation experiments indicate that the mutagenic agents were all able to act as very potent inactivating or killing agents in *B.fragilis*. This indicates that it is unlikely that the mutagenic agents are being inactivated or reduced in an obligate anaerobe such as *B.fragilis*. It is interesting to note that the two mutagenic agents which were tested (EMS and UV radiation), stationary phase cells were more resistant to the effects of the mutagens. One possible explanation for the low yield of mutants obtained is that the bactericidal effect of the various mutagens is enhanced in *B.fragilis* in the presence of oxygen, causing a rapid drop in viability, so that very few sublethally damaged cells survive. Unfortunately this facet was not appreciated at the time that the program aimed at isolating mutants was carried out, and unfortunately the effects of oxygen enhancement of mutagenic inactivation was not investigated. However subsequent work on inactivation by UV radiation has shown that a marked oxygen effect is present, and the implications are discussed in Part 2 of this work.

The lack of success in isolating specific amino acid auxotrophic mutants in the two strains of *B.fragilis* used in this study, suggests that these strains may be unable to take up amino acids. These findings are in keeping with a number of other reports which indicate that some strains of *Bacteroides* are only able to utilize amino acids poorly, or not at all.

CHAPTER III

COLONIAL PHASE VARIATION IN THE *BACTEROIDES FRAGILIS* GROUP

3.1 INTRODUCTION

Of the more than 300 species of anaerobic bacteria which are known to occur as part of the normal flora of the human body, there are only about 6 species which are consistently isolated from clinical infections (Finegold *et al.*, 1977). Virulence appears to be an important factor in the selection of the limited number of species which are commonly found in septic processes. Virulence in bacteria has been attributed to a number of factors. Many pathogens are known to produce specific virulence enhancing substances such as enzymes and toxins. In other bacteria the virulence factors have been shown to be part of the normal components of the outer surface of the cell. In many Gram-negative bacteria complex lipopolysaccharides (LPS) which form part of the cell wall are able to act as endotoxins. In other groups of bacteria the possession of an antiphagocytic polysaccharide capsule has been shown to confer virulence on the organisms.

Amongst the *Bacteroidaceae* a considerable amount of interest has been focused on the nature of the surface layer of the cell, both in relation to the possible role of these components as virulence factors, and in relation to the immunological responses of the host to infections by *Bacteroides*. The nature of the surface antigens of the *Bacteroidaceae* and their relationship to immune responses, immunoprophylaxis, and the serological detection and classification of members of this group has been reviewed recently by Hofstad (1979).

Of all the anaerobes *B.fragilis* has been the most frequently isolated from clinical specimens (Washington, 1971; Thadepalli *et al.,* 1973 a, b; Chow & Cuze, 1974; Swenson *et al.,* 1974; Mathias *et al.,* 1977; Polk & Kasper, 1977). The distribution of *B.fragilis* has been shown to be markedly different in the normal flora and infected sites. In the colon (the usual source of *B.fragilis* in septic infections) *B.fragilis* only accounts for *c.* 0.5% of the colonic microflora, and is greatly outnumbered by the species *B.distasonis, B.thetaiotaomicron,* and *B.vulgatus.* In clinical isolates *B.fragilis* is the dominant form and accounts for *c.* 80% of all isolates (Jones & Fuchs, 1976; Polk & Kasper, 1977; Lindberg *et al.,* 1979). The predominance of this species in clinical isolates strongly suggests that it possesses unique virulence properties.

Encapsulation in *B.fragilis* was reported by a number of the early workers (Hofstad, 1979) and both the role of the capsule and LPS, in virulence has been investigated in *B.fragilis.* Kasper *et al.* (1979) concluded that although the LPS of *B.fragilis* has some of the chemical and physical properties of an endotoxin it lacks the biochemical properties of a true endotoxin and is thus unlikely to play any major role in the virulence of this organism. In *B.fragilis* the relationship between the presence of a capsule and virulence still appears to be unclear. Kasper *et al.*(1977) reported that the possession of a capsule was unique to *B.fragilis,* and Onderdonk *et al.* (1977) reported a correlation between the presence of a capsule and virulence. In a further study they demonstrated that abscess formation in rats could be directly attributed to the capsular polysaccharide (Kasper *et al.,* 1979).

However the findings of a number of other workers have not been in full agreement with these workers. Beerens *et al.*(1963) reported varying degrees of encapsulation in members of the genus *Eggerthella (Bacteroides)*. Babb & Cummins (1978) supported these earlier observations when they found that the presence of capsules was not restricted to *B.fragilis*. They reported some degree of encapsulation was also displayed by most strains of *B.vulgatus*, *B.thetaiotaomicron* and *B.ovatus* as well as *B.fragilis*. Lindberg *et al.* (1979) also found that the strains of *B.distasonis*, *B.ovatus*, *B.thetaiotaomicron*, *B.uniformis* and *B.vulgatus* which they examined all showed varying degrees of encapsulation. They found that *c.* 80% of *B.fragilis* and *c.* 30-40% of *B.ovatus*, *B.thetaiotaomicron* and *B.uniformis* and *c.* 5% of *B.vulgatus* and *B.distasonis* cells were encapsulated when examined under the light microscope using the India-ink staining method, and under the electron microscope using the ruthenium red staining method. Their results also indicated that the proportion of encapsulated strains isolated from faecal material and clinical infections were about the same. In addition the presence of a capsule has also been reported in a strain of *B.thetaiotaomicron* which exhibited colonial variation (Burt *et al.*,1978) and the presence of a capsule has also been reported to be associated with pathogenicity in a strain of *B.melaninogenicus* (Takazoe *et al.*, 1971).

Some of the differences in findings reported by Kasper, Onderdonk and their co-workers and those reported by other workers, appears to be due to differences in opinions as to what constitutes a capsule. Kasper (1976) defined the capsule in *B.fragilis* as an antigenic high molecular weight polysaccharide material external to the multilayered cell wall which could be made visible in the electron microscope by staining with ruthenium red, and which was about 0,2 μm in width. This external polysaccharide was found to be species specific for *B.fragilis* (Kasper *et al.,* 1977) and it is thought that it may play a role in *in vivo* adhesion (Kasper *et al.,* 1978). The polysaccharide of Kasper may represent a cell surface antigen rather than a true capsule (Babb & Cummins, 1978; Hofstad, 1979). The use of sodium deoxycholate to separate the antigen from other membrane components also supports this suggestion. Babb & Cummins (1978) found that there was no direct correlation between the presence of a capsule and virulence, when assessing the presence of encapsulated cells, in the more classical sense (as referring to structures external to the cell wall and easily visible under the light microscope).

A number of workers have reported that the presence of a capsule(as determined using a light microscope) may only occur in a proportion of the cells in a population. Babb & Cummins (1978) found that the proportion of encapsulated cells varied among the different strains but represented *c.* 10% or less of the total cell number for most isolates. However, in a few strains all the cells in the population were encapsulated. In an investigation of pseudolysogeny in *Bacteroides* phages,Booth *et al.* (1979) reported that the phenomenon appeared to be due to the formation of a large capsule by a small proportion of the cells in the culture (*c.*10% of the cells) and that this capsule interfered with the attachment of the phage to the cell. In a report by Burt *et al.*(1978) a strain of *B.thetaiotaomicron* was described which exhibited colonial variation associated with the presence and absence of encapsulated cells. This strain segregated 2 unstable colonial variants at high frequency, the one colony type was characterized by being circular, entire, white, opaque, mucoid and convex in appearance and the majority of cells from this type of colony were found to be encapsulated. The other colony type was circular, entire, greyish, semitranslucent, with a fried egg type appearance and most of the cells from this colony type were found to lack capsules. Both colony types were unstable and always gave some colonies of the opposite type when plated out. These workers also reported that the encapsulated cells were resistant to a phage (β1) which was able to infect non-encapsulated cells.

In addition to the presence of surface layers, micro-capsules, true capsules and slime layers in many species of *Bacteroides*, the presence of a glycocalyx consisting of loose fibrous materials and extensive thin fibrils has been reported to occur in strains of *B.asaccharolyticus* and *B.melaninogenicus* isolated from oral infections (Woo *et al.*, 1978; Lai & Listgarten, 1978; Woo *et al.*, 1979).

The numerous reports documenting a variety of different surface structures in different species of *Bacteroides* suggests that the possession of such structures is a common feature of this group. The nature and role of these surface layers appears to be complex and their role in cell adhesion, cell-cell interactions, virulence and phage resistance are still far from being completely understood.

In this study the presence of capsules or slime layers in the various strains of *Bacteroides* was determined, and their relationship to colonial phase variation, pseudolysogeny, phage resistance and bacteriocin resistance was investigated.

3.2 METHODS

3.2.1 EXAMINATION OF COLONIAL MORPHOLOGY

In strains showing strong colonial variation the phenomenon was clearly visible on examination of the culture plates (Plate 3.1 F). Strains showing weak colonial variation were examined using a stereoscopic zoom microscope with a magnification of x10 to x45 (Bosch & Lomb), and fitted with a built in illumination source which allowed viewing with both transmitted and reflected light. In addition weak colonial variation was also enhanced by staining the colonies with a variety of common microbiological stains. Methylene blue was found to be one of the most suitable stains for this purpose.

3.2.2 LIGHT MICROSCOPY

Both agar- and broth-grown cells were examined using a Zeiss RA microscope fitted with phase contrast optics and equipped with a photomicrographic camera. To demonstrate the presence of an extracellular layer the India-ink wet-mount technique was used. Earlier studies in which cells were examined for the presence of extracellular material using an electron microscope to visualize thin sections stained with ruthenium red gave similar results to those obtained with the light microscope. As this technique was time consuming and appeared to give limited additional information it was not used in this investigation.

3.2.3 THE DYNAMICS OF COLONIAL PHASE VARIATION

The two types of colonies present in a number of stains were cloned *c*. 30 times over a 2 month period. The proportion of cells giving rise to colonies of the opposite colony type was determined by suspending cells obtained from a single colony in 10 ml of Ringer solution and plating onto BHI agar immediately. The frequency of segregation of the two cell types from each colony type was determined by plating after 18 h growth in BHI broth.

3.2.4 ENVIRONMENTAL EFFECTS ON COLONIAL VARIATION

Selected *Bacteroides* strains were grown under different growth conditions to assess the effect on colonial variation and the production of extracellular material. Plate cultures were incubated at 20, 25, 30, 37 and 42°C to determine the effect of temperature. The effect of pH was determined by growing cells on BHI agar plates buffered at various pH levels (pH 5,8; 6,0; 6,4; 7,4; 7,8; 8,0) (See Appendix II 4.3.1). The effect of oxygen was determined by incubating agar plates in the presence of varying amounts of air (1, 2, 5, 10, 15, 20 and 25% air). The agar plates were placed in an anaerobic jar which was connected to a manometer and the jar and manometer were flushed with an anaerobic atmosphere for 20 min. The required proportion of the anaerobic atmosphere was then evacuated and replaced with a corresponding volume of air. The effect of light was determined by inoculating and incubating the agar plates either in the absence or presence of light.

The effect of different nutrients was determined by plating selected strains of *Bacteroides* onto a variety of different nutrient media with or without additional nutrient supplements.

3.2.5 INDUCTION, CURING AND MUTAGENESIS

Attempts were made to try and alter the frequency of colonial variation and capsule production in a number of different strains of *Bacteroides* by subjecting them to a variety of mutagenic and plasmid curing agents. Exponential phase cultures of each colony type were exposed to treatments with UV radiation and NTG as described in 2.2.7. The two types of cells were also grown in the presence of increasing concentrations of mitomycin C (0,01; 0,1; 1; 10 μg ml^{-1}) in both liquid and solid culture media. The effect of acriflavine, ethidium bromide or sodium-dodecyl sulphate (SDS) was determined by inoculating buffered BHI broth (pH 7,6) containing doubling dilutions of acriflavine (1 - 32 μg ml^{-1}), ethidium bromide (0,5 - 10 μg ml^{-1}) or SDS (0,05 to 5%) with diluted exponential phase cells obtained from the two colony types. The cells were plated onto BHI agar plates after 24 h incubation.

3.2.6 ANTIBIOTIC SENSITIVITY OF THE TWO COLONY TYPES

The sensitivity of the two colony types from various *Bacteroides* strains to the following antibiotics was determined by the sensitivity disc method: ampicillin, clindamycin, chloramphenicol, erythromycin, fusidic acid, metronidazole, mynocycline, nitrofurantoin, nicene, novobiocin, septrin, tetracycline.

3.3 RESULTS

3.3.1 COLONIAL PHASE VARIATION IN THE *BACTEROIDES FRAGILIS* GROUP

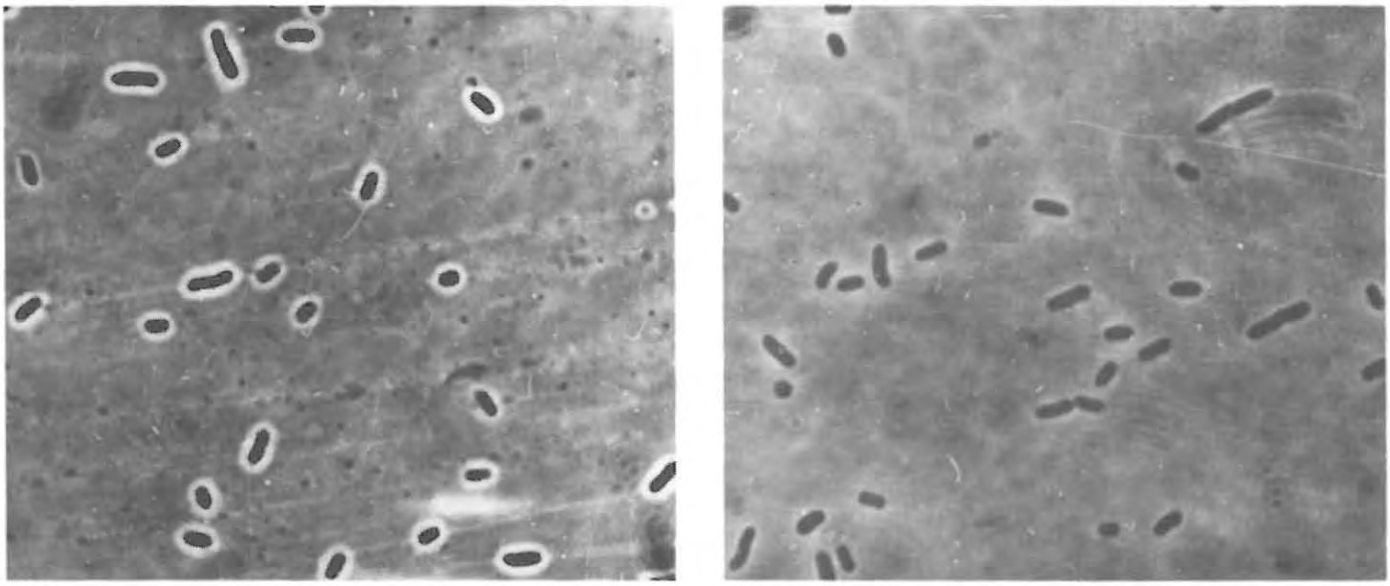
A strain of *B.thetaiotaomicron* which segregated 2 unstable colonial variants at high frequency was reported by Burt *et al.* (1978). In addition to this strain which was included in this investigation (RV4) the other 35 strains of *Bacteroides* were also examined to see if similar colonial variations occurred. Three of the strains of *B.fragilis*, RV1, RV2 and RV3 were found to show clearly visible variations in colonial morphology (Plate 3.1 C, D, E, F) which were similar to those observed in the original strain of *B. thetaiotaomicron*. There was no easily viable colonial variation present in the other 32 strains, but when they were examined with the aid of a stereoscopic microscope variations in colonial morphology were apparent in all the strains. However, in these strains the contrast between the two colony types was very much less, and could only be distinguished with difficulty, without the aid of a microscope and suitable illumination. The contrast between the two colony types could be enhanced by staining with various common microbiological stains.

Observations of a number of other species of bacteria under the same conditions did not reveal any variations in colonial morphology and it was concluded that the distinctive type of colonial variations observed in the *Bacteroides* strains was a specific characteristic of this group.

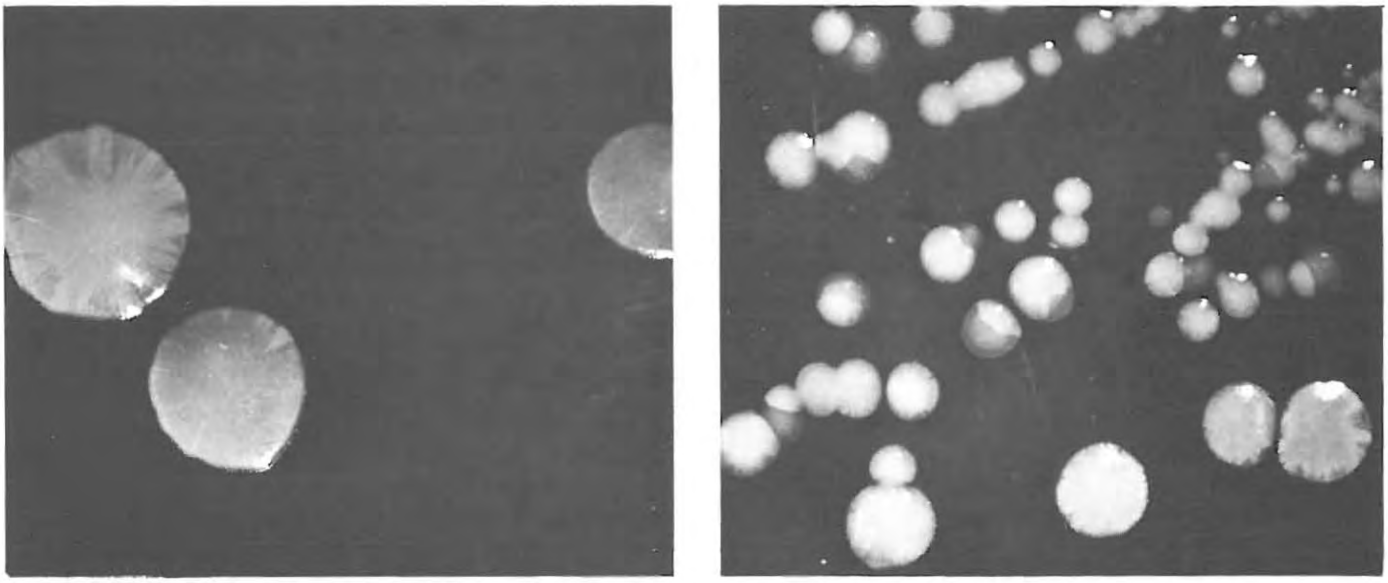
In the 4 strains showing strong colonial variation, two distinct colony types could be distinguished. One type of colony was circular, entire, white, opaque and mucoid in appearance (type I colonies). The other type was circular, entire, greyish, semi-translucent and non-mucoid (type II colonies). Type I colonies tended to be slightly larger in diameter and more convex than the type II colonies. If no attempts were made to select for one or other colony type then the two colony types occurred in approximately the same numbers (50:50) when plated out onto agar plates.

In the other 32 strains showing weak colonial variations the difference in the two colony types was very much less marked. Both colony types

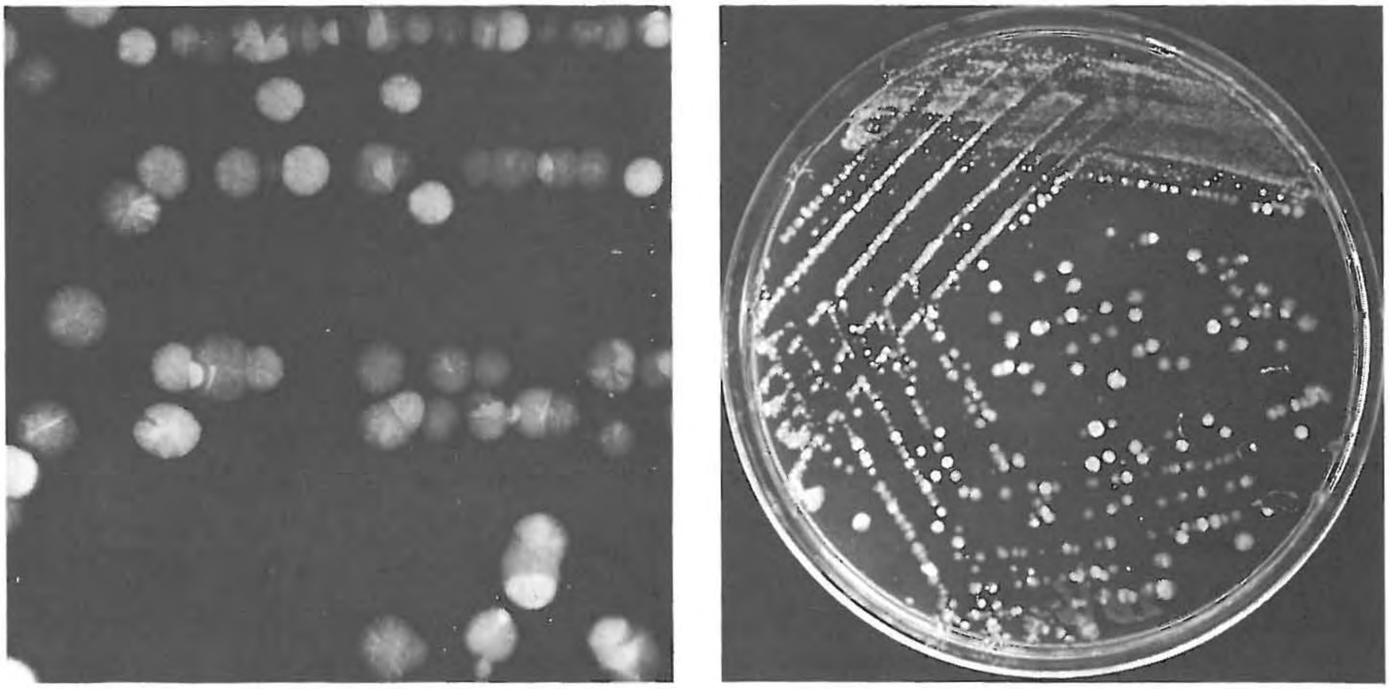
PLATE 3.1



(A & B) CAPSULATED AND NON-CAPSULATED CELLS



(C & D) COLONIES SHOWING PIE SECTORING



(E & F) COLONIES SHOWING PHASE VARIATION were similar to type II colonies, but one was slightly more opaque than the other. In some strains a third more opaque mucoid colony was also present on occasions. In strains showing weak contrast in colony types the proportion of the more opaque colonies tended to be very much lower, and in many of the strains only accounted for *c.* 10% of the total number of colonies. In other strains however they occurred in higher proportions, sometimes making up more than 50% of the total number of colonies. Observations were made on all 36 strains at *c.* 4 weekly intervals over an 18 month period, and the intensity and proportion of the opaque colonies were recorded. Both the intensity and the proportion of the more opaque (type I) colonies in the individual strains was found to remain more or less constant over the whole of the 18 month period. Although minor variations in both the intensity and proportion of the colonies did occur, each strain was found to display a characteristic pattern of colonial variation (Table 3.1).

3.3.2 COLONIAL VARIATION AND THE PRODUCTION OF EXTRACELLULAR MATERIAL

Cells from both colony types of all 4 strains showing strong colonial variations were examined for the presence of extracellular material using the India-ink wet-mount technique. In all 4 strains the mucoid colonies were found to consist almost entirely of encapsulated cells. In most mucoid (type I) colonies more than 90% of the cells were encapsulated whereas cells from type II colonies were mostly non-capsulated with less than 10% of the cells possessing capsules (Plate 3.1, A, cells from type I colonies and B, cells from type II colonies). The capsules of cells obtained from a single population showed quite marked variations in size. Most cells possessed capsules which were between 1 and 1,5 times the diameter of the cell. However cells with capsules ranging from very small up to 4 times the cell

TABLE 3.1

Colonial variation and the production of extracellular material in *Bacteroides* strains monitored over a period of 18 months.

Bacteroides strains	Contrast of type I colonies	Proportion of type I colonies (%)	Production of capsule or slime layer
RV1	++++	40-60	++++
RV2	++++	40-60	++++
RV3	++++	40-60	++++
RV4	++++	40-60	++++
RB2	++/+++	40-60	±/+
R4	++	40-100	±
RS3	+/++	30-50	++
PS6	+/++	10-50	+
RS4	+	20-40	+
R2	+	20-40	+
R3	+	20-40	+
PS2	+	10-40	±
PB2	+	10-40	+
RB4	+	10-40	+
RS7	+	10-40	+
P1	+	10-30	+
PB3	+	10-30	+
R1	+	10-30	+
RB1	+	0-30	+++
RS6	+	0-30	±
PS7	+	0-10	±
PS1	±/+	20-40	±
RS8	±/+	10-40	++
PB1	±/+	10-30	+
PS5	±/+	0-30	++
RBS2	±/+	0-30	+
RBS3	±/+	0-30	+
RS5	±/+	0-30	±
RB3	±/+	0-10	+
RBS1	±/+	0-10	±
RS1	±	10-30	+
PS3	±	0-30	+
PS8	±	0-30	++
RB5	±	0-30	±
PS4	±	0-15	++
RS2	±	0-15	+

Contrast of type I colonies.

++++ = Clearly visible
+++-+ = Just visible
±/+ = Visible only under microscope
± = Not always visible under microscope

Production of extracellular material.

++++ — ± = Very abundant to very sparce.

diameter were usually present. In liquid cultures *c.* half the cells in the populations were found to be encapsulated. The capsular material obtained from these 4 strains was found to be a polysaccharide (Dettman & Jones, unpublished data).

In all of the other 32 strains of *Bacteroides* examined some extracellular material was observed to be present in association with the more opaque colony types. In some strains clearly defined, apparently rigid capsules were observed to be present but in lower proportions than were present in cells from the strains showing strong colonial variation. In many strains however the cells were surrounded by a slime layer rather than a clearly defined capsule. In some strains this slime layer adhered to the cells while in others the cells were embedded in a fibrous matrix of slime but individual cells did not have any closely adherent slime layers. In some strains the production of a copious slime layer resulted in an increased viscocity in broth cultures. In strains which normally only produced slime layers encapsulated cells were observed to be present under certain conditions.

3.3.3 DYNAMICS OF COLONIAL VARIATION

Although two clearly distinguishable colony types were always present in strains showing strong colonial variation, many colonies which were intermeidate between the two types were also present. A complete spectrum in colony types ranging from type I to type II colonies occurred and segmentation and pie sectoring was common (Plate 3.1 C, D, E, F). When colonies which were apparently of one type or the other were plated out, they always gave rise to at least 10% of colonies of the other type, indicating that segretation between the two colony types occurred at high frequency. This was also borne out by the fact that the number of intermediate colony types showed an increase with age. After several days incubation most isolated colonies which had originally appeared to be of one type, developed segments within the colony which contained cells of the opposite cell type (Plate 3.1 C and D). In broth the segregation frequency of the two cell types was found to be *c.* 1 x 10^{-2} variants per generation.

In many of the strains showing weak colonial variation the proportion of opaque colonies in the population was very much lower and in some strains these colonies constituted less than 10% of the population. In other strains they constituted *c.* 50% of the population. The frequency of segregation in the different strains was fairly stable. However, in the one strain of *B. distasonis* included in this study the proportion of opaque colonies varied from *c.* 50% to *c.* 100% from time to time. The cells from the opaque colonies in this strain were associated with the production of a slime layer but no capsules were present.

3.3.4 EFFECT OF DIFFERENT GROWTH CONDITIONS ON COLONIAL VARIATION AND THE PRODUCTION OF EXTRACELLULAR MATERIAL

Four strains of *Bacteroides* showing strong colonial variation (RV1, RV2, RV3, RV4) and 4 strains showing weak colonial variation (RB1, RB2, PS1, R4) were grown under different growth conditions to assess the effect on colonial variation and the formation of extracellular material.

Overnight cultures (15-18 h) of the 8 strains of *Bacteroides* were streaked onto BHI agar plates and incubated in a CO_2 atmosphere containing an increasing percentage of air. Individual strains showed slight variations in sensitivity to the presence of small amounts of oxygen in the atmosphere. However, all strains showed a marked decrease in growth in atmospheres containing 10% or more air. The effect of oxygen on colonial variation was difficult to assess. The growth of isolated individual cells, spread onto the surface of agar plates was inhibited by 2-5% air in the atmosphere. In atmospheres containing higher percentages of air, growth only occurred in the area of the initial inoculum where large numbers of cells had been deposited close together on the surface of the agar. The concerted effect of the large number of cells appeared to be sufficient to produce a local reduction of the environment and allow growth to occur. Colonies which developed in the area of the initial inoculum were very mottled giving the appearance of a marked increase in the frequency of colonial variation. However this effect appeared to be mainly or entirely due to the fact that these colonies had developed as a result of the confluent growth of many cells, rather than resulting from an increased segregation frequency due to the effect of oxygen. At low concentrations of air (1-2%), where no loss in viability occurred, there was no increase in the segregation frequency of the colonial variants. In atmospheres containing a small percentage of air the amount of extracellular material was similar to the amount produced under anaerobic conditions. In atmospheres containing a higher percentage of air, the amount of extracellular material present in the surviving colonies was increased.

All 8 strains tested showed diminished growth when incubated at 20 and 42°C. The colonies which did develop in the area of the initial inoculum showed a mottled appearance which was interpreted as being due to the decrease in growth rather than to an increased frequency of segregation. No loss in viability occurred when cells were incubated at 25, 30 and 37°C. All the strains except R4 showed a small increase in the proportion and intensity of type I colonies with increasing temperature (Table 3.2). The production of capsules in the type I colonies did not appear to be affected by temperature, but an increase in the amount of extracellular slime was observed at 37°C.

All 8 strains grew over the pH range tested (5,8-8,0) but all strains showed a decrease in growth above pH 7,0. All the strains except RV showed a decrease in the proportion and the intensity of type I colonies with increasing pH values (Table 3.3).

Higher pH values also resulted in a slight decrease in both the size and the number of capsules produced by cells from type I colonies and there was also a decrease in the amount of extracellular slime produced.

Light was found to have no effect on either colonial variation or the production of extracellular material.

The growth of the 8 strains was tested on 4 different nutrient media (minimal media, peptone media, blood agar base, and BHI agar) both with and without the additional supplement of 10% sucrose, 10% glucose and KCl, all of which are known to increase capsule production in *Klebsiella* and *Pneumococcus* (Wilkinson, 1958). Both the proportion and the intensity of type I colonies were found to increase with the addition of sucrose and KCl to the media and also with the improved nutrient status of the media (Table 3.4). The size and the number of capsules present in cells from type I colonies was only very slightly increased on the supplemented or enriched media. However, the amount of extracellular slime showed a substantial increase.

TABLE 3.2

The Effect of temperature on the proportion of type I colonies.

Strains of *Bacteroides*	Incubation temperature ^{o}C				
	20	25	30	37	42
RV1	-	30%	40%	45%	-
RV2	-	30%	40%	45%	-
RV3	-	35%	45%	55%	-
RV4	-	40%	45%	50%	-
RB1	-	30%	35%	40%	-
RB2	-	20%	20%	25%	-
PS1	-	5%	10%	10%	-
R4	-	100%	100%	100%	-

- = Growth too poor to make an assessment.

TABLE 3.3

The effect of pH on the proportion ype I colonies.

Strains of *Bacteroides*	pH of BHI plates					
	5,6	6,0	6,4	7,4	7,8	8,0
RV1	60%	60%	55%	50%	-	-
RV2	60%	60%	55%	50%	-	-
RV3	65%	60%	50%	40%	20%	-
RV4	50%	50%	50%	20%	20%	-
RB1	60%	50%	50%	45%	20%	-
RB2	40%	30%	35%	20%	10%	-
PS1	10%	10%	5%	5%	<5%	-
R4	100%	100%	100%	100%	100%	-

- = Growth to poor to make an assessment.

TABLE 3.4

The effect of different nutrient media (with and without supplements) on the contrast and proportion of type I colonies, and the production of encapsulated cells in these colonies in the strains RV1, RV2, RV3 and RV4.

Culture media	Contrast of type I colonies	Proportion of type I colonies (%)	Percentage of encapsulation in type I colonies
MM	+	10-20	70-90
MM + ADD	+	20-35	>90
PEP	+	5-20	70-90
PEP + ADD	++	25-55	>90
BAB	+	10-25	>90
BAB + ADD	+++	25-45	>90
BHI	+++	20-50	>90
BHI + ADD	++++	30-70	>90

MM = Minimal media
PEP = Peptone agar
BAB = Blood agar base
BHI = BHI agar
ADD = glucose, sucrose and KCl additives.

Contrast.
\+ = Weak
++ = Moderate
+++ = Strong
++++ = Very strong

3.3.5 ATTEMPTS TO INDUCE OR 'CURE' COLONIAL VARIATION

Exposure of selected strains of *Bacteroides* to mutagenic or inducing agents such as UV radiation, NTG and mitomycin C had no effect on the frequency of segregation of colonial variants. Growth of cells in media containing acriflavine, ethidium bromide, and SDS did not affect the segregation frequency of colonial variants. Attempts to isolate stable mutants of type I or type II colonies were unsuccessful.

3.3.6 ANTIBIOTIC SENSITIVITY AND COLONIAL VARIATION

Type I and type II colonies obtained as segregants from 8 strains of *Bacteroides* (RV1, RV2, RV3, RV4, PS4, PS8, RS6, R4) were tested against 12 different antibiotics to determine whether the sensitivity of the two colony types differed. The two colony types showed the same sensitivity to the antibiotics, except in one case where in 4 of the 8 strains a slight decrease in the sensitivity of type I colonies to the antibiotic septrin was observed.

3.4 DISCUSSION

In a study on encapsulation of *Bacteroides* species Babb & Cummins (1978) found that encapsulation in *Bacteroides* was not limited to *B.fragilis* as previously reported (Kasper *et al.*, 1977; Onderdonk *et al.*, 1977), but that most strains belonging to the *B.fragilis* group showed some degree of encapsulation. They found that the proportion of encapsulated cells varied among the different strains, and within single cell suspensions. In most strains only 10% or less of the cells were encapsulated. They found that in many strains the capsules were well defined and quite rigid but some strains possessed a copious slime like layer instead of a true capsule. Capsule production was not found to vary significantly with the age of the culture, incubation, temperature or pH and was not found to be significantly affected by several organic and inorganic nutrients. In this present study on the production of extracellular material by 36 strains belonging to the *B.fragilis* group the findings did not differ from those of Babb & Cummins (1978) to any significant degree, except in one aspect. Babb & Cummins (1978) reported that they observed no difference in colonial morphology relative to encapsulation, and microscopic examination of isolated colonies revealed both encapsulated and non-encapsulated cells in porportions similar to those in broth.

A strain of *B.thetaiotaomicron* which segregated two unstable colonial variants at high frequency and which showed a correlation between colonial morphology and encapsulation was reported by Burt *et al.* (1978). In addition to this strain which was included in this study, a further 3 strains of *B.fragilis* which exhibited similar segregation of colonial variants at high frequency were investigated. All 3 strains showed a correlation between the absence or presence of encapsulated cells and the type of colonial morphology. The segregation frequencies and the dynamics of colonial variation in these 3 strains were found to be very similar to those found in the original strain of *B.thetaiotaomicron* (Burt *et al.*, 1978). In addition all other 32 strains belonging to the *B.fragilis* group were found to display a weak colonial variation which was also found to be correlated with either the production of capsules or an extracellular layer of slime. This observation suggests that this phenomenon may be fairly widespread amongst members of the *B.fragilis* group. The lack of correlation between capsule production and colonial morphology reported by Babb & Cummins (1978) may be due to the fact that in most strains variations in colonial morphology can only be observed with the aid of a microscope.

The high frequency of segregation suggests that the rapid variation between the two colonial phases is not due to mutation. This is borne out by the fact that mutagenic agents such as UV radiation and NTG do not affect the frequency of segregation. Neither does the fluctuation between the two colony types appear to be due to physiological adaptation to changes in the environment. Exposure to a number of different growth conditions only produced small variations in the proportion and intensity of the two colony types and had little effect on the production of extracellular material by some of the cells in the population. As the segregation occurs in both directions at the same frequency, it seems unlikely that capsule production and colonial variation could be controlled by extrachromosomal elements. The lack of any effect by agents such as acriflavine, ethidium bromide and SDS, which are known to 'cure' cells which harbour plasmids, supports this view.

There are numerous reports of variation in colonial and morphology occurring in different species of bacteria which have been found to be associated with alteration in virulence, phage susceptibility or other changes in physiology. In many cases the colonial variants are known to be stable and appear to have resulted from mutations which affect the production of cell surface components such as LPS or other cell wall components or extracellular components such as the capsule or glycocalyx. Many of these mutations give rise to changes in colonial morphology from mucoid to smooth or from smooth to rough colonies. The absence or presence of pili and the ability of cells to form long chains may also give rise to changes in colonial morphology.

In addition to stable changes in colonial morphology there are a number of reports which mention unstable colonial variation (Kellogg *et al.*, 1968; Willis, 1969; Thorley, 1976). However, little appears to be known about the mechanism responsible for these changes.

Besides colonial morphology a number of other surface structures are known to undergo variation from one state or phase to another at high frequencies. The change from piliated to non-piliated cells in the population in known to occur at a high frequency in a number of groups of bacteria and may be accompanied by changes in colonial morphology (Duguid & Gillies, 1958; Duguid & Wilkinson, 1961; Thorley, 1976; Brenner *et al.*, 1977). The best studied system of rapid phase variation is that of flagella variation in *Salmonella*. This phenomenon has been well elucidated since its divcovery more than 50 years ago (Ilno, 1977; Silverman & Simon, 1977). Most strains of *Salmonella* are known to produce two types of flagella which can be agglutinated by different antisera. Switching between the two flagella phases was found to occur at frequencies 10^2 to 10^3 times higher than the mutation frequencies (Stocker, 1949). Each flagella type was found to be specified by separate structural genes H1 and H2. The switching between the 2 genes was found to involve a change at the DNA level as the state or phase of expression of the H2 gene could be transduced. The switching mechanism was further elucidated when the use of molecular cloning techniques allowed the preparation of relatively large amounts of the specific DNA which carried the phase switch (Zieg *et al.*, 1977). They isolated a derivative of Col E1 which contained the H2 gene which still showed phase variation. The DNA appeared to be a mixture of molecules carrying the phase 2 genes in both the on and the off state. Denaturing and reanealing of the DNA indicated that the switch was a result of an inversion at the DNA level. Inversion bubbles were seen in 5 to 10% of molecules in electronmicrographs. The inversion sequence was found to be adjacent to the H2 gene and was 750 bases long. It was concluded that phase variation in *Salmonella* was determined by a specific control region adjacent to the H2 gene which can undergo an inversion. In one configuration it allows the H2 gene and H1 repressor to be transcribed and in the inverted position it prevents H2 and H1 repressor transcription. This so called 'Flip Flop' control mechanism has been found in a number of other regulation processes involving phase variations such as in the *gal* operon (Saedler *et al.*, 1974) and in phage Mu (Bukhari, 1976). In all cases regulation is known to be brought about by insertion sequences or transposons (Bukhari *et al.*, 1977). It is interesting to note the insertion of such elements is mediated by non-specific illegitimate recombination which is independent of *rec* A function. Colonial variation in *Bacteroides* appears to bear a number of similarities to flagella phase variation in *Salmonella* and some sort of 'flip flop' control mechanism seems to provide a plausible explanation for the phenomenon.

Colonial variation in *Bacteroides* appears to occur as a direct result of a certain proportion of the cells in the population producing extracellular material. Evidence reviewed in the introduction to this chapter indicates that the cell surface of many strains of *Bacteroides* may have a number of extracellular components associated with it. At present the function of these surface components is not fully understood. There is evidence to suggest that they may play important roles as defense mechanisms against phagocytosis, phage infection and adverse environmental conditions. They have also been implicated in cell adhesion and dispersal, and may play an important role as virulence factors and antigens in infection. The possession of surface layer components appears to be widespread amongst the *Bacteroides* but it seems probable that in many strains extracellular material may only be present in a certain proportion of the cells in the population. In many strains less than 10% of the cells are encapsulated, however, the proportion of encapsulated variants may increase under certain conditions by selection. The fact that the possession of surface components may be variable in some strains, should be considered when using colonial morphology, serotyping, and phage typing for the identification and classification of *Bacteroides* strains. These variations may also play an important role in the virulence and antigenicity of *Bacteroides* strains in clinical infections. The relationship of colonial phase variation to phage-resistance and pseudolysogeny and possibly bacteriocin-resistance is discussed in the following two chapters.

CHAPTER IV

BACTERIOPHAGES OF THE *B.FRAGILIS* GROUP

4.1 INTRODUCTION

A limited number of studies on the bacteriophages of *Bacteroides* have been reported. The first report of a phage which was specific for *Bacteroides* was made by Sabiston & Cohl (1969). They isolated a virulent phage from sewage which was mostly active against *B.distasonis* species.

The isolation of a number of phages from clinical sources which were active against *Ristella pseudoinsolita (B.fragilis)* was reported by Prévot *et al.* (1970). These authors also presented evidence of a lysogenic strain of *R.pseudoinsolita.*

Two bacteriophages which were specific for *B.fragilis* were isolated from sewage by Nacescu *et al.* (1972). These phages lysed 23 out of the 68 strains tested. In addition these workers reported isolating 8 phages from mixed cell cultures, presumably as a result of induced prophages. However they were not able to induce phages in pure culture.

Brandis *et al.* (1972) reported on the further characterization of one of the two phages isolated from the sewage. This phage (ϕA1) was found to belong to Bradley's morphological group B and exhibited no unusual properties with respect to latency, heat and pH stability or adsorption kinetics.

A number of phages specific for *B.fragilis* were isolated from animal sera by Keller & Traub (1974). In addition these authors characterized a phage which could establish a carrier state with the host bacterium.

Silver *et al.* (1975) reported on bacteriophage-like particles which were observed in thin sections of *B.fragilis* cells associated with larger spherical bodies 300 nm in diameter. Phage heads were always present in association with these bodies, and the intact phages appeared to belong to Bradley's group B. These structures were presumed to be temperate phages although no sensitive strain was found and it was not possible to induce the phage. The significance of the association with the spherical bodies is not known.

The properties of a phage (β1) which was able to establish a carrier state with *B.thetaiotaomicron* was described by Burt & Woods (1977). This phage which was used in transfection studies, produced a high proportion of defective particles in the infected host cell.

Booth *et al.* (1979) isolated 67 phages from sewage which were specific for a number of species of *Bacteroides*. A study of these phages showed that they belonged to 4 distinct morphological types, only one of which had been described previously. They also observed that pseudolysogeny (phage carrier state) occurred in the majority of the phages. These workers investigated the feasibility of developing a system which could be used as a rapid method of identification of *Bacteroides* species.

The possible occurrence of lysogenic phages in *Bacteroides* has been suggested in two of the earlier reports discussed, and is supported by the observations of Silver *et al.* (1975) of phage-like particles associated with spherical bodies in *B.fragilis*. However Booth *et al.* (1979) reported that all of their phages were virulent, and they were unable to detect lysogeny in 83 strains of *B.fragilis*. Thus true lysogeny would appear to be rare or difficult to induce in members of the *Bacteroides* group. However pseudolysogeny appears to be extremely common in this group.

The term pseudolysogeny or the carrier state has been given to the group of phage host relationships which are neither truly lysogenic or virulent. The precise nature of the pseudolysogenic state is not well defined and appears to vary from group to group. In most pseudolysogenic conditions reported so far, the phage appears to exist in the cytoplasm of its host without any mechanism to ensure its regular segregation to daughter cells at division. An equilibrium is found to exist between sensitive and resistant bacteria, maintenance being achieved by phage infection of the sensitive bacteria in the population (Baess, 1971).

The phage-host relationship in pseudolysogeny is distinct from that observed during P1 infection of a lysogenic host. In this case, stable lysogens occur despite the fact that the phage is not integrated into the host chromosome but exists as an autonomous replicon in the cytoplasm (Ikeda & Tomizawa, 1968). Replication of the prophage is under strict control and non-lysogenic segregants are rare. A similar situation has been reported for prophage N15, which appears to have an extrachromosomal location (Ravin & Schulga, 1970).

Pseudolysogens are most easily distinguished from stable lysogens by treatment of the host cell with phage-specific antiserum (Anderson & Cowles, 1958; Romig & Brodetsky, 1961; Bott & Strauss, 1965; Baess, 1971). This eliminates the phage-carrier state, by preventing reinfection of sensitive cells, causing an enrichment of non-pseudolysogenic segregants. A similar effect is obtained by growth in the absence of a required phage adsorption factor (Clarke, 1952). Curing by acridine dyes is indicative of cytoplasmic factors and this has been used as a method for distinguishing between lysogens and pseudolysogens (Arditti & Coppo, 1965; Eklund *et al.*, 1971). Another generally accepted feature of pseudolysogeny is that the phages are non-inducible (Baess, 1971;

Bott & Strauss, 1965). However Arditti & Coppo (1965) reported an inducible carrier state for *Bacillus megaterium*. Certain bacteriophages have been reported to exist as plasmids in the cytoplasm of the host cell e.g. λ infection of *E.coli* - *S.typhosa* hybrids (Baron *et al.*, 1970; Falkow & Baron, 1970). The characteristics of these phages appear to be similar to those of carrier phages in that not all bacteria were found to possess phage and the phages were not inducible.

Kawakami & Landman (1968) observed that antiserum treatment of the *B.subtilis* SP-10 carrier state gave large fluctuations in the intracellular repressor levels as well as rapid curing of the carrier state. They proposed that the carrier state is maintained entirely by reinfection, and that no replication of the phage occurs in the latent state. The segregation of non-lysogenic cells becomes possible as soon as the latent period of a phage exceeds the generation time of the host.

In a number of cases the carrier state has been found to be associated with changes in the bacterial phenotype. Li *et al.* (1961) observed that all phage-containing colonies were lactose-positive and all phage free colonies were lactose-negative. This alteration was thought to be due to a phage-coded enzyme which altered the permeability of the host cells, allowing lactose to diffuse into the cell in the absence of a permease system. Jones *et al.* (1962) reported that in *Brucella abortus* the phage carrier state was associated with a change in colonial morphology. Colonies containing phage were found to be white and sticky while those not containing phage were blue-grey. They suggested that these alterations were also due to a phage enzyme which releases some of the mucopeptides in the cell walls resulting in the sticky consistency.

Despite the absence of any stable association with the bacterial chromosome, pseudolysogenic phage have been shown to bring about transduction (Thorne, 1962; Bott & Strauss, 1965).

Transduction has proved to be one of the most useful systems for mapping bacterial genes, especially fine structure mapping. Transduction was first demonstrated in *Salmonella typhimurium* with the phage P22 by Zinder & Lederberg (1952) and it has subsequently been reported in a large number of other bacterial species. Transducing phages can be divided into 2 classes according to the range of host markers which they are able to transfer.

In generalized transduction any host genes may be transferred. A transducing lysate may be produced either by infection of sensitive bacteria, or, by induction of lysogenic cells, and contains phage particles which have a low transducing activity for each host gene. Upon infecting a recipient bacterium, the fragment of bacterial chromosome which enters the cell, may undergo recombination with the host cell genome. Recombination between the host cell genome and the exogenote appears to be brought about by the same enzyme systems responsible for recombination during conjugation and transformation.

In specialized transduction the phage is able only to transduce a small number of bacterial genes, which lie adjacent to the prophage attachment site. The bacterial markers contained in the specialized transducing particles are obtained by illegitimate pairing between host DNA adjacent to the prophage and the prophage, during the process of induction (Campbell, 1962). An important characteristic of specialized transduction is that the transducing phage can only be obtained by induction of a lysogen. When the transducing particles infect another bacterial cell lysogenization may occur, yielding transductants which are partial diploids. The mechanisms of marker incorporation in specialized transduction therefore appear to differ from those involved in generalized transduction. Phage P22 which is capable of both generalized and specialized transduction has been found to be capable of specialized transduction, but not generalized transduction in *rec*$^-$ mutants (Wing, 1968).

The distinction between generalized and specialized transduction, however, may not always be clearly defined. Luria *et al.*(1960) reported generalized transducing phages which yielded heterogenote transductants and Chakrabarty & Gunsalus (1969) showed that transductants of mandelate genes in *Pseudomonas putida* could yield stable or unstable transductants, depending on the degree of homology between incoming and recipient DNA as well as on the residual phage function in the transducing phage. Woods & Thompson (1975) reported generalized transduction of 6 auxotrophic markers in *Achromobacter* strains which resulted in unstable transductants, characteristic of transduction by lysogeny.

In most instances it has been temperate phages which act as the vectors for transduction. However, both virulent and pseudolysogenic phages are also known which are capable of bringing about transduction (Zinder & Lederberg, 1952; Thorne, 1962; Bott & Strauss, 1965). Recently Wilson *et al.* (1979) described a multiple mutant of T4 that displayed the property of generalized transduction, and which is capable of transferring *E.coli* genes with frequencies higher than those observed for other transducing phages.

In this study the isolation of a number of phages specific for members of the *B.fragilis* group was undertaken, with the primary aim being to try and isolate a transducing phage. In addition attempts were made to try and induce any lysogens which might have been harboured in the strains of *Bacteroides* which were investigated. As a number of *Bacteroides* phages have already been characterized (see 4.1), and these did not appear to exhibit any extraordinary or peculiar properties, no attempt at any detailed characterization of the phages was undertaken.

In addition to the transduction experiments the phages were also used in the study of pseudolysogeny and colonial phase variation. Phages were also used in attempts to obtain phage resistant host cell mutants and phage mutants for use in marker rescue experiments (9.3.4). The phages also proved to be extremely useful tools in the study of the physiological responses of *B.fragilis* to UV radiation (Chapter 9).

4.2 MATERIALS AND METHODS

4.2.1 ISOLATION OF BACTERIOPHAGES

Sewage and pooled faecal samples were screened for the presence of phage. Sewage samples were collected from various sites (raw sewage, primary and secondary settling tanks, anaerobic digester, etc.) from a sewage treatment plant in Grahamstown. Pooled stool samples obtained from a hospital laboratory, and diluted in phosphate buffer were also screened for the presence of phage. Individual or pooled samples were clarified by centrifugation at 2 000 x *g* for 20 min. The supernatants were then either filtered through a 0,45 µm membrane filter fitted with a pre-filter, or treated with 10% chloroform. The filtrates were then screened for the presence of phage by spotting onto double overlay plates (Adams, 1959) which had been seeded with 0,2 ml of overnight cultures of a selection of the *Bacteroides* strains used in this investigation. Each phage was purified by 3 successive single plaque transfers. Attempts to induce lysogenic phages in the 36 *Bacteroides* strains used in this study were made, using the methods described by Parisi & Tarbot (1974) and Siddiqui *et al.* (1974).

4.2.2 PHAGE PROPAGATION AND PREPARATION OF HIGH TITRE LYSATES

High titre phage lysates were obtained either by confluent lysis techniques on BHI agar plates or by broth lysis. Higher titres of all the phages were consistently obtained using the confluent lysis technique. Overnight cultures (15-18h) of the specific host cell (1,0 ml) and 0,5 ml of the phage, suspended in T2 buffer, were added to 15 ml of soft BHI agar and overlayed onto 6 BHI agar plates and incubated at $37^{\circ}C$ overnight. The soft agar was then scraped off into 30 ml of T2 buffer, mixed with a vortex mixer and allowed to stand for 2 h at room temperature. The agar suspension was then centrifuged at 10 000 x *g* for 20 min and the agar free supernatant removed by means of a Pasteur pipette. The supernatant containing the phage was stored at $4^{\circ}C$ over 10% (v/v) chloroform. The stability of the phages in a number of different buffers held under a variety of conditions was determined. For phage lysates prepared by the broth lysis technique the method described by Burt & Woods (1977) was used.

4.2.3 PHAGE ASSAY

The plaque-forming titres of the phage samples were determined using the soft-agar overlay method described by Adams (1959). All manipulations were performed aerobically. The phages were routinely diluted in T2 buffer. BHI agar plates were overlayed with 2,5 ml of BHI soft agar (0,7%) which had been seeded with 0,2 ml of the assay organism. The plates were then incubated anaerobically at $37^{\circ}C$ overnight.

4.2.4 EFFECT OF THE AGE OF THE HOST CELL ON THE EFFICIENCY OF PLATING OF THE PHAGES

The efficiency of plating (e.o.p.) of the phages on the host cell, strain PS1, at different stages in the growth cycle was determined using the assay technique described in 4.2.3.

4.2.5 HOST RANGE AND HOST SENSITIVITY

Phage titres were adjusted to give a titre of 10^8 p.f.u. ml^{-1}. Ten fold dilution series of the phages were prepared and spotted onto a series of overlay plates which had been seeded with the 36 *Bacteroides* strains used in this investigation. In addition the susceptibility of 12 other species of bacteria to these phages were tested *(Fusobacterium varium, Fusobacterium mortiferum, Escherichia coli B, Escherichia coli* K12, *Shigella boydii, Salmonella typhimurium, Proteus vulgaris, Klebsiella pneumoniae, Enterobacter aerogenes, Serratia marcescens, Clostridium perfringens, Staphylococcus aureus).*

4.2.6 ATTEMPTS TO OBTAIN TRANSDUCTION

High titre lysates (10^9 - 10^{11} p.f.u. ml^{-1}) of 12 phages (FC 1-6, FT 1-6), were prepared according to the method described in 4.2.2 and used to examine the potential for transfer of several genetic markers in *B.fragilis*. These phages were propagated in *B.fragilis* host strains PS1 and P1, both wild type and rifampicin, erythromycin, vancomycin and nitrofurantoin resistant mutants were used for phage propagation. The recipient strains (PS1, P1) included both wild type and several auxotrophic mutants derived from the above strains. In addition the transducing potential of 9 phages which were specific for the single strain of *B.thetaiotaomicron* included in this survey were also determined. The phages were propagated on both the wild type and rifampicin resistant host cells (RV4) and either the wild type or a nicotinamide requiring auxotroph of RV4 were used as the recipients.

In a series of experiments to test for the potential for transduction, phages were added at a m.o.i. ranging from 0,01 to 5, to both exponential and stationary phase cells. Adsorption was allowed to occur under both anaerobic and aerobic conditions for varying periods of time before plating, using a variety of adsorption media. In some experiments the recipient cultures, and or, the phages, were exposed to varying fluences of UV radiation under aerobic conditions, before adsorption.

4.2.7 PSEUDOLYSOGENY AND PHAGE RESISTANCE

The isolation of phage resistant mutants from 3 strains of *B.fragilis* (PS1, PB1 and P1) was attempted using 3 different phages FC1, FC2 and FC3. The phages were added at a m.o.i. of 5 to overnight cultures of the host cell which had been mutagenized with NTG as described in 2.2.7. The phage infected cells were overlayed in soft agar (3 ml) onto the surface of 20-30 BHI agar plates and incubated overnight at 37°C. Any isolated colonies present in the overlays in which confluent lysis had occurred were picked and re-tested for phage resistance. After each subculture isolated colonies were tested for phage susceptibility and the presence of the original phage.

4.3 RESULTS

4.3.1 THE ISOLATION OF PHAGES

A total of 17 phages specific for *B.fragilis* were obtained from sewage samples collected at weekly intervals over a period of several weeks. In addition 4 phages which were specific for the single strain of *B.thetaiotaomicron* used in this investigation were also isolated from these samples. A further phage which was specific for this strain was isolated from pooled faecal samples obtained from a hospital laboratory. Of the 17 phages specific for *B.fragilis*, 5 were lost during subculturing or storage, or were discarded due to difficulties encountered in propagating them.

All attempts to induce lysogenic phages which may have been present in the 36 strains used in this investigation, gave negative results.

4.3.2 GROWTH CHARACTERISTICS OF THE *B.FRAGILIS* PHAGES

The *B.fragilis* phages were routinely propagated on strain PS1. Individual phages showed variations in the maximum titres that were obtained by the techniques used. With some of the phages, titres of 10^9 p.f.u. ml^{-1} could only be obtained with difficulty, with others titres of between 10^9 and 10^{11} p.f.u. ml^{-1} were readily obtained. With all the phages consistently higher titres were obtained using the confluent lysis technique. Stationary phase cells gave higher phage yields than exponential phase cells.

The 12 *B.fragilis* phages were found to be highly stable under the conditions tested. Little or no drop in phage titre occurred in lysates which were stored over chloroform at 4°C for over a year. All the phages appeared to be virulent. None of the phages caused visible lysis in broth culture of the susceptible host strains which were tested. On overlay plates,6 of the phages (FC 1-6) produced clear plaques and phages FT 1-6 produced small turbid plaques on *B.fragilis* PS1. Plaques varied from clear to extremely turbid depending on the particular host strain on which it was plated.

4.3.3 VARIATIONS IN THE e.o.p. OF PHAGES PLATED ON CELLS OF *B.FRAGILIS* STRAIN (PS1) OF INCREASING AGE

Plaque counts done on the same phage samples were found to show considerable variation depending on the age of the indicator cells used in the assay. An increase in the number of p.f.u. ml^{-1} was found when stationary phase cells were used instead of exponential phase cells. A number of different experiments were conducted using phages FC 1-6 to determine the e.o.p. of these phages when plated onto PS1 host cells of increasing age. The results of one such experiment (using

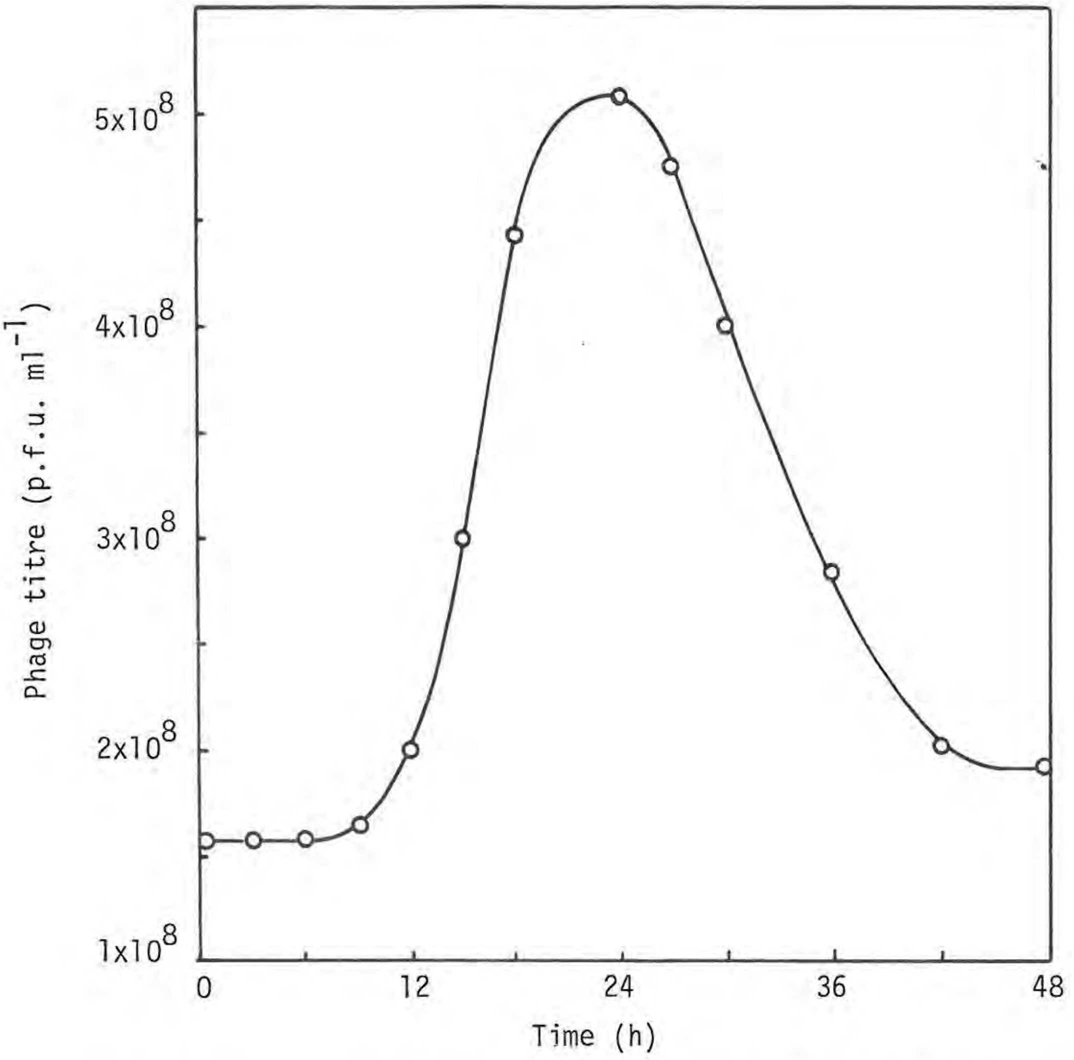


Fig. 4.1. Variation in the efficiency of plating of phage during the growth cycle of *B.fragilis* PS1.

phage FC2) is shown in Fig. 4.1. In all the experiments a consistent increase in the e.o.p. in stationary phase cells occurred with all 6 phages. The actual increase varied from phage to phage and also varied from experiment to experiment with the same phage. However in all cases the e.o.p. increased from the beginning of the stationary phase up until about 24 h. and then gradually decreased again back to the initial level.

4.3.4 HOST RANGE AND HOST SPECIFICITY

None of the *Bacteroides* phages had any effect on the bacteria belonging to other species of bacteria. Each phage was tested for its host range against all 36 strains of *Bacteroides* used in this investigation. The 5 phages which were specific for the single strain of *B.thetaiotaomicron*, showed no activity against any of the other 35 strains of *Bacteroides*. Of the 12 phages isolated on *B.fragilis* host cells,all infected at least 3 other strains of *B.fragilis*. The lytic patterns of 10 of the phages is shown in Table 4.1. The full lytic patterns for phages FT4 and FT5 were not done. Even though a very limited number of strains were used only two of the phages showed the same lytic pattern. The groups of susceptibility patterns were quite similar, indicating that some of the phages may be closely related. Four strains of *B.fragilis* (PS1, PS2, PB1, P1) were susceptible to all 12 of the phages, 15 of the *B.fragidis* strains were sensitive to at least one of the 10 phages tested and 16 were not susceptible to any of the 10 phages. None of the other 5 strains belonging to the 3 other species of *Bacteroides* were susceptible to any of the phages.

TABLE 4.1

Patterns of phage lysis shown by the 15 strains of *B.fragilis* showing phage sensitivity.

Strains of *B.fragilis*	*B.fragilis* phages									
	FC6	FC3	FC4	FC1	FT2	FC2	FC5	FT3	FT1	FT4
PS1	+	+	+	+	+	+	+	+	+	+
PS2	+	+	+	+	+	+	+	+	+	+
PB1	+	+	+	+	+	+	+	+	+	+
P1	+	+	+	+	+	+	+	+	+	+
PB2	+	+	+	+	+	+	+	-	-	-
PS3	+	+	+	+	+	+	+	-	-	-
PB3	+	+	+	+	+	+	+	-	-	-
PS4	+	+	+	+	+	-	-	-	-	-
PS5	+	+	+	+	+	-	-	-	-	-
PS6	+	+	+	-	-	-	-	-	-	-
PS7	+	+	+	-	-	-	-	-	-	-
PS8	+	-	-	-	-	-	-	-	-	-
RB1	-	-	-	-	+	+	+	+	-	-
RS4	+	+	-	-	-	+	-	-	-	-
RB2	-	-	-	-	-	-	+	-	-	-

- = Not sensitive
+ = Sensitive

NB Complete host range sensitivities were not done on phages FT5 and FT6.

4.3.5 ATTEMPTS TO OBTAIN TRANSDUCTION

With both the *B.fragilis* system and the *B.thetaiotaomicron* system, no transduced clones were detected under any of the experimental conditions used.

4.3.6 PSEUDOLYSOGENY AND PHAGE RESISTANCE

Nine phages specific for *B.thetaiotaomicron* (4 isolated by Burt 1977 and 5 isolated during the course of this investigation) were tested for their ability to infect type I and type II colonies of *B.thetaiotaomicron* (RV4). Exponential phase cultures of 30 colonies of each colony type were tested for resistance to the 9 phages by spotting high titre lysates (10^{10} p.f.u. ml^{-1}) on to soft agar overlays seeded with cells from type I or type II colonies. All 30 of the type I colonies containing encapsulated cells were resistant to the 9 phages and all 30 of the type II colonies containing non-encapsulated cells were sensitive to the 9 phages. None of these 9 phages produced visible lysis in broth. A number of resistant colonies were isolated from turbid plaques or plates showing confluent lysis for all 9 phages and were inoculated into BHI broth. All the isolates produced phage with titres ranging from 10^5 to 5×10^8 p.f.u. ml^{-1} after 9 h incubation. One phage resistant clone of each phage type was isolated and tested for the stability of phage resistance and pseudolysogeny over 3 consecutive subcultures. After each cloning the colonies were tested for phage production and phage resistance. The number of colonies producing phage after each cloning varied from 30 - 90% and the number of colonies resistant to phage varied from 50 - 100%.

None of the *B.fragilis* strains which were sensitive to the 12 phages isolated during this study showed strong colonial variation. The plaque morphology varied from clear to very turbid depending on the phage and the host cell. In broth culture none of the phages caused visible lysis, suggesting that a phage carrier state similar to that described by Keller & Traub (1974) and Booth *et al.* (1979) was occurring. The relationship between the host strain P1 and the phages FC1, FC2 and FC3 was investigated. This strain showed weak colonial variation with between 10 and 30% of type I colonies present on plating. Between 5 and 10% of the cells in the population were encapsulated. Cells from 9 h cultures which had been infected with phage were plated out and individual colonies were picked into broth and tested for phage production after 9 h incubation. Between 60 and 90% of the colonies produced phage. Spread plates of infected clones gave viability counts similar to those obtained with uninfected cultures. Spot tests on soft agar overlays seeded with phage producing clones were not susceptible to the phages and these clones were found to contain 90 - 95% of encapsulated cells. The phage producing clones continued to produce phages after further subculture.

The phages FC1, FC2 and FC3 all produced clear plaques when plated on the host strains PS1, PB1 and P1. Attempts were made to isolate phage-resistant mutants after mutagenic treatment of these 3 strains. Isolated colonies present in the area of confluent lysis on overlay plates were picked and tested for phage-resistance and the ability to produce phage. A high proportion (60 - 95%) of these colonies were found to be resistant to phage and continued to produce phage particles on subculture. These colonies showed an altered colonial morphology being much more opaque, and examination of the cells from these isolates showed that a high proportion of these cells were encapsulated.

Only one of the three strains was observed to produce encapsulated cells in uninfected cultures, the other two strains (PS1, PB1) showed very weak colonial morphology and a small proportion of the cells appeared to produce a loose slime layer. The encapsulated clones were maintained in culture for several weeks but eventually all the cells reverted to the non-encapsulated form. No stable non-encapsulated phage-resistant mutants were isolated using this technique.

4.4 DISCUSSION

A number of phages specific for strains of *B.fragilis* were isolated from sewage. Four of the strains of *B.fragilis* used in this study were sensitive to all the *B.fragilis* phages which were isolated, while 16 strains of *B.fragilis* were not susceptible to any of the phages. None of the other species of *Bacteroides* were sensitive to these phages. The e.o.p. of these phages was found to increase after the end of the exponential phase of growth, and continued to rise for several hours before dropping again. The reason for the increase in the e.o.p. is not known and experiments to investigate this phenomenon are underway. Nine phages specific for the single strain of *B.thetaiotaomicron* were also isolated (4 by Burt, 1977). One of these phages was isolated from a human faecal sample, the other 8 were isolated from sewage. None of the other 35 strains of *Bacteroides* were susceptible to these phages.

All the phages were virulent and attempts to induce lysogenic phages proved unsuccessful. Although the presence of lysogenic phages in *Bacteroides* has been suggested by some of the earlier work, the systematic search for lysogenic phages by Booth *et al.*(1979) and our own lack of success indicates that either lysogenic phages are not common in *Bacteroides* or else are not easily induced or propagated under the experimental conditions used.

Both the *B.fragilis* phages and the *B.thetaiotaomicron* phages were used in attempts to obtain transduction without success. In experiments with a number of *Bacteroides* phages Booth *et al.* (1979) were also unable to detect transduction. They suggested that the failure to do so may have been due to the fact that they were unable to isolate any temperate phages. Plasmid transfer presumably via conjugation appears to occur quite readily in *Bacteroides* (see Chapter I) and transfection of phage DNA leading to the recovery of virulent phage particles has been shown to occur in *B.thetaiotaomicron* (Burt & Woods, 1977). However to date there is no evidence for the transfer of stable chromosomal markers in *Bacteroides*. The establishment of a system capable of mediating such transfer would be of considerable value in the future studies of this group.

Keller & Traub (1974) were the first to report that a stable carrier state was readily developed in *B.fragilis* phage-host systems. They found that in broth cultures phage production occurred coincidently with bacterial growth. They ascribed the simultaneous production of both phage and bacteria to the continuous development of phage-sensitive cells from a phage-resistant population. Most cells in the carrier cultures were found to be phage-resistant but continued to segregate phage-sensitive variants which supplied host cells for the maintenance of phage replication. Cultures of the phage-susceptible clones were found to contain no detectable phage, whereas cultures of phage-resistant clones continued to produce phage. They found that phage production in these clones began at the same time as active bacterial growth started, and that phage production outstripped bacterial growth over an 18 h period. Their results suggested that a rapid rate of alternation between the two phenotypes occurred in the population. Cultivation of the phage carrier strain in media containing phage antiserum eliminated the phage carrier state. The cured cultures yielded a mixture of phage-sensitive and phage-resistant clones. Before phage infection they failed to detect any phage-resistant phenotypes in the culture. However, after curing with antisera *c*. 80% of the population was phage-resistant, and this proportion remained constant after several subcultures. Phage-sensitive clones showed between 1 and 8% segregation of phage-resistant clones. Their results indicated that the phage was adsorbed with equal efficiency by both phenotypes and they postulated that pseudolysogeny was most likely to result through variations in intracellular immunity. A search for cytoplasmic elements proved negative.

A carrier state very similar to that reported by Keller & Traub (1974) was found to occur in the majority of phage-host systems in *Bacteroides*, which were investigated by Booth *et al*. (1979). They found that the presence of a thick capsule around some of the cells in the host strain appeared to render these cells resistant to phage infections and was responsible for perpetuating the carrier state. Non-infected host strains were found to contain *c*. 5% encapsulated cells whereas most cells in infected populations were found to be encapsulated. About 80% of these cells were found to be phage-resistant after subculture and continued to produce phage on serial transfer. After being cured by growth in media containing phage antisera they became susceptible to the phage again.

In most species of bacteria which are known to produce capsules a correlation between phage resistance and the production of a capsule has been reported (Jones *et al.*, 1962; Bernheimer & Tiraby, 1976; Grant & Riemann, 1976; Poxton & Sutherland, 1976). Burt *et al.* (1978) reported a strain of *B.thetaiotaomicron* which segregated two unstable colonial variants at high frequency. Type I colonies containing encapsulated cells (see Chapter 3) were found to be resistant to the phage β1, while type II colonies which contained non-encapsulated cells were susceptible to the phage. Phage-resistant clones continued to produce phage and the carrier state was found to persist through a number of subcultures. Both phage-resistant encapsulated cells and phage-sensitive non-encapsulated cells were found to segregate cells of the opposite type at high frequency.

In this present study, type I colonies containing encapsulated cells were found to be resistant to all 9 phages isolated from this strain. Phage-resistant clones isolated after infection all continued to produce phage. These clones of phage-resistant encapsulated cells continued to segregate non-encapsulated, phage-sensitive cells at a high frequency thus supplying host cells for the continued propagation of the phage.

None of the strains of *B.fragilis* which were sensitive to the phages isolated in this study, showed strong colonial phase variation. In most cases infected host strains exhibited a carrier state which appeared to be identical to that reported by Booth *et al.* (1979). In the uninfected state these host strains had a very low proportion of encapsulated cells. After phage infection, phage-resistant clones showed pseudolysogeny and a high proportion of encapsulated cells. In some strains encapsulated cells were not present in uninfected cells but these cells did produce an extracellular slime layer. Individual cells did not seem to be phage-resistant but many of the cells contained within a matrix of slime were not susceptible to phage-infection, and such clones of cells continued to produce phage. When these strains were treated with mutagenic agents before being infected with phage, phage-resistant colonies were found to develop within the zones of clear lysis on overlay plates. These phage-resistant clones were found to contain a high proportion of encapsulated cells and exhibited pseudolysogeny. Capsule production was found to persist through a number of subcultures, but eventually all these strains reverted to the non-encapsulated variant.

In all the examples discussed above the carrier state appeared to be due to capsule formation by some cells in the population which interfered with the attachment of the phage to the cell wall. The continued presence of the phage (in the carrier state) appeared to be due to the infection of sensitive cells which segregated from the phage-resistant clone at high frequency. Evidence suggests that the continued presence of phage in such clones was due to the association of the phage particles with the extracellular material, rather than the maintenance of the phage within the cytoplasm of the cells, although this possibility has not been ruled out. The presence of this phenomenon in phage-host cell systems in *Bacteroides* was found to complicate the isolation of stable phage-resistant mutants and attempts to isolate such mutants were not successful.

CHAPTER V

BACTERIOCIN PRODUCTION IN THE *BACTEROIDES FRAGILIS* GROUP

5.1 INTRODUCTION

Many bacterial strains liberate proteinaceous toxins called bacteriocins, which are active only against closely related strains of bacteria. Bacteriocin production has been discovered in many genera, both in Gram-negative and Gram-positive bacteria. However in the latter group a wide variety of inhibitors and toxins are produced, so that it is difficult to decide which of them can usefully be called bacteriocins.

The most extensively studied group of bacteriocins are those produced by *E.coli* and other closely related enterobacteria such as *Shigella sonnei*, which have been termed colicins. The literature on colicins has been reviewed by a number of authors (Fredericq, 1957, 1963; Reeves, 1972; Hager, 1973; Holland, 1975; Luria,1975; Hardy,1975, 1978) and much of the following information on colicins has been obtained from these reviews.

In all colicins of natural origin investigated so far, the genetic determinants of colicin production have been found to occur on plasmids. These colicinogenic (Col) factors can be divided into 2 major groups according to their size and their ability to transfer themselves. Only the larger group II plasmids (mol.wt.*c*. 70×10^6) are self transmissible, and virtually all carry the genes for conjugation and plasmid transfer in addition to colicin production. Group II Col factors are usually only present in one or two copies per cell. The smaller group I plasmids (mol.wt.*c*. 5×10^6) are usually maintained at about ten copies per cell and are not transmissible. Col factors can recombine with other plasmids, suggesting that F-like Col factors probably arose by the integration of Col and F factors. Col factors which are non transmissible by their own means, can in some cases be transmitted when the cell also harbours a transmissible plasmid as well. It is possible that in some cases, joint transfer results from a transient integration of the two plasmids. In most cases however, the data suggests that the non transmissible plasmid is defective in one or more gene functions required for conjugation and the missing functions are supplied by the active plasmid.

Colicins are proteins with molecular weights ranging from *c*. 27 000 to 90 000. Although in most cases colicins vary widely in their amino acid composition, there are a number which have similar compositions, indicating a common origin. Colicins have been classified into about 20 different types on the basis of their action on colicin resistant bacteria (Fredericq, 1957).

Normally, in any population only a small proportion of Col$^+$ cells produce detectable amounts of colicin (usually between about 0,01 and 10% of the population). The apparent repression of colicin production suggests an obvious parallel between colicinogenic bacteria and lysogenic bacteria. In both cases the phenomenon can be induced by agents such as mitomycin C (Mit C) and UV radiation. The mechanisms of colicin induction are unknown but in some cases they may be induced by processes similar to those involved in the induction of lysogens (See 10.1). In the case of colicin induction, complete cell lysis does not occur, as it does in lysogeny. In a number of colicinogenic strains (e.g. Col E1 and Col E2) induced cells are unable to form colonies although colicin is released for several hours. Cell death does not necessarily occur as a result of colicin production in all cases.

A distinctive feature of colicins is their narrow action spectrum. Their selective action is due to the need for specific protein receptor sites in the outer envelope of the sensitive bacteria. These receptor sites have been found to be proteins or glycoproteins which, in most cases, have other functions such as in membrane transport, and in several cases they also act as receptors for bacteriophages. The first step in the killing of cells by a colicin is the binding of the colicin to receptors on the external surface. Adsorption of colicin does not produce irreversible changes leading to cell death unless the cytoplasmic membrane is energised. Only if membrane energy is available do colicin treated cells enter stage II which leads to cell death. Colicins do not all have the same mode of action in bringing about cell death, and different colicins kill cells by affecting widely different biochemical targets. For example, colicin E3 is known to inactivate ribosomes, the actual colicin or part of it, probably penetrates into the cell and is responsible for inactivating the ribosomes. A number of colicins such as El, K and I bring about cell death by disrupting many of the energy dependent reactions in the cytoplasmic membrane. In other colicins such as E2 the chromosomal DNA is the target for endonucletic degradation (Schaller & Nomura, 1976).

When a Col factor is transferred into a colicin-sensitive strain, this strain then becomes immune to the action of the corresponding colicin even though the cell still possesses colicin receptors and can still adsorb the colicin. In the cases studied so far, immunity appears to be brought about by low molecular weight proteins which bind to the bacteriocin protein and inactivate it. Genes for the immunity protein are probably present on all Col plasmids.

There are 2 types of mutants which are insensitive to colicins, those in which the receptors have been lost or altered, and those which still adsorb colicin onto the receptors but which are nevertheless immune. Tolerant mutants may be insensitive to a number of colicins which have different actions, indicating that there is a common step after adsorption which is common to all these colicins.

Although much more is known about colicins than about other groups of bacteriocins, many groups of bacteria are known to produce bacteriocins. Amongst the Gram-negative bacteria most other genera of *Enterobacteriaceae* are known to produce bacteriocins as well as other genera such as *Pseudomonas, Yersinea, Neisseria* and *Vibrio*. Several of the bacteriocins produced by these bacteria resemble the colicins in that they are inducible by mitomycin C and UV irradiation.

The earliest reports of bacteriocin production in *Bacteriodes* species were by Beerens & Baron (1965) and Beerens *et al.* (1966). Podhaisky & Reinhold (1970) investigated bacteriocin production in 70 strains of *Bacteroides* belonging to the *fragilis* group, originating from clinical material. They found bacteriocins were produced by 61 of the strains and only 1 *B.fragilis* strain was resistant to all the bacteriocins produced. The bacteriocins produced by strains of *Bacteriodes* isolated from human faeces and the role of these strains in the bacterial ecology of the colon was investigated by Booth *et al.* (1977). This study was carried out on an individual who was part of the National Aeronautics and Space Administration mock Skylab flight and faecal samples were monitored over a 25 week period. Four bacteriocin producing strains were isolated, only one of which was found to persist over the 25 week period. This particular bacteriocin was partially characterized. The bacteriocin producing strains were found to coexist in the colon with a larger population of non-bacteriocin producing, but bacteriocin-sensitive strains of *Bacteriodes*.

In this study bacteriocin production in a number of strains was discovered while screening for lysogenic phages. The pattern of bacteriocin production and bacteriocin-sensitivity of the strains being used was determined, and attempts were made to isolate bacteriocin-resistant mutants from sensitive strains for use as markers in the transduction experiments.

In addition the relationship of bacteriocin resistant colonies and capsule production was investigated. One of the bacteriocins was subsequently partially characterized and its mode of action determined (Mossie *et al.*, 1979). This bacteriocin was found to be unusual as the primary event in its mode of [illegible] is the inhibition of RNA synthesis. Rifampicin resistant mutants isolated from a bacteriocin sensitive strain (PS1) were found to be resistant to the action of the bacteriocin with the exception of one mutant which was found to be hypersensitive.

5.2 METHODS

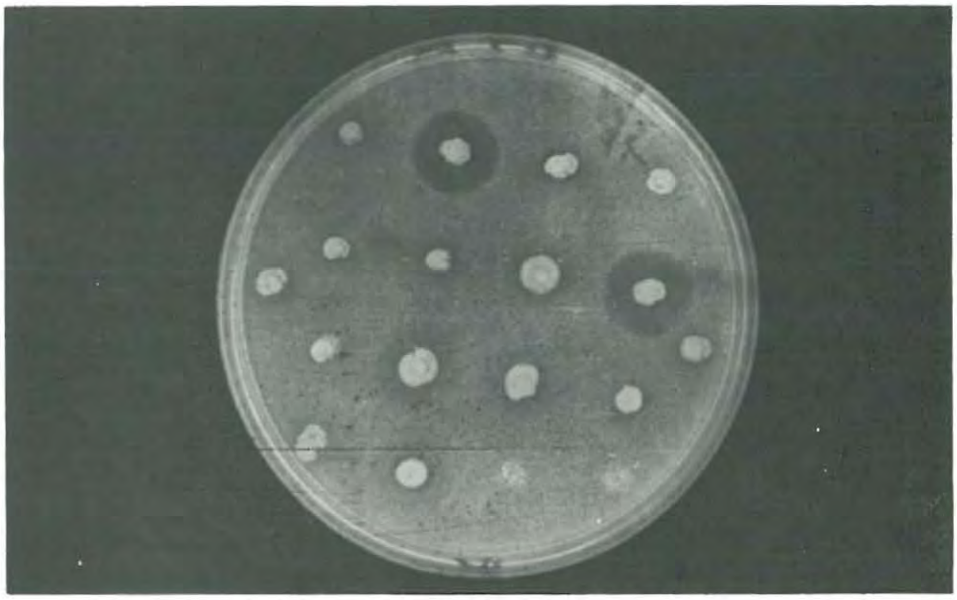
5.2.1 DETECTION OF BACTERIOCIN PRODUCTION AND SENSITIVITY

The 36 strains of *Bacteroides* used in this investigation were tested for bacteriocin production and sensitivity using modifications of techniques described by Mayr-Harting *et al.* (1972). Overnight cultures (15-18 h) of the 36 strains were spotted onto plates which had been overlayed with 3 ml of BH1 soft agar inoculated with 0,2 ml of overnight cultures (15-18 h) of the test stains, or alternatively onto dried BH1 agar plates. Inoculations were made by means of a replicator which could inoculate 18 stains onto each test plate. The plates were then incubated for 48 h at 37°C under anaerobic conditions. The colonies which developed on the plates without the overlays were then killed by inverting the plates over containers of chloroform for 30 min and then standing for 20 min to allow the chloroform to evaporate. The plates were then overlayed with 3 ml of BH1 soft agar which had been inoculated with the test stains as in the previous procedure. The plates were examined for zones of inhibition after 24-48 h incubation (Plate 5.1 A).

5.2.2 INDUCTION OF BACTERIOCIN PRODUCTION

In order to test for the induction of bacteriocin production by Mit C the screening procedures described in 5.2.1 were carried out on BH1 agar plages containing 0,1; 1 and 10 μg ml^{-1} Mit C. To test for induction by UV radiation assay plates inoculated by the first screening technique described in 5.2.1 were exposed to fluences of 15 Jm^{-2} of UV radiation before incubation. In addition the effect of Mit C and UV radiation on the induction of increased levels of bacteriocin production in 2 stains, PB1 and RB3, were determined. Doubling concentrations of Mit C (0,1 - 1,6 μg ml^{-1}) were added to early exponential phase cultures (OD 0,2) of the 2 strains and they were incubated and assayed after 6 and 12 h incubation. Exponential phase cells of the same 2 strains were suspended in Ringer solution and irradiated with fluences of 15 and 30 J m^{-2}. The cells were then

PLATE 5.1



(A) ZONES OF INHIBITION PRODUCED BY BACTERIOCIN.

(B) A BACTERIOCIN ASSAY PLATE

centrifuged and inoculated into BHI broth and the bacteriocin titres assayed after 6 and 12 h incubation.

5.2.3 DEMONSTRATION THAT THE EFFECT WAS DUE TO A BACTERIOCIN

Material from the inhibition zones of all 20 of the producer strains was picked and tested for the presence of phage by the standard plaque assay (Adams, 1959). The host range of all 20 producer strains was tested against 12 strains of non related bacteria.

5.2.4 BACTERIOCIN ASSAY

Indicator strains (0,1 ml of PS1 or RS1) were added to 10 ml of molten BHI agar (1% agar w/v) held at $45-50^{o}$C. The agar was mixed and poured into sterile petri dishes. After they had solidified the plates were perfused with an anaerobic atmosphere for 20 min. Wells of 5 mm diameter were then punched into the agar using a sterile cork borer. The supernatants of the test cultures were harvested by centrifugation at 10 000x *g* for 10 min at 4^{o}C and stored over 10% chloroform at 4^{o}C to kill any residual bacteria. Doubling dilutions of supernatants from the test cultures were made and 25 μl of each dilution was added to the wells. The assay plates were incubated under anaerobic conditions overnight (15-24 h). The bacteriocin activity in arbitrary units (AU) was defined as the reciprocal of the highest dilution which gave a definite zone of inhibition (Plate 5.1 B).

5.2.5 DETERMINATION OF OPTIMAL CONDITIONS FOR BACTERIOCIN PRODUCTION

Two of the producer strains (PB1 and RB3) were grown in MM, peptone yeast extract medium, and BHI broth at 30^{o} and 37^{o}C and the supernatants were assayed for bacteriocin production at the end of the exponential phase of growth.

5.2.6 THE RELEASE OF EXTRACELLULAR BACTERIOCIN

Chloroformed supernatants from two producer strains (PB1 and RB3) were assayed over a 72 h period to determine the level of extracellular bacteriocin.

5.2.7 THE LOCALIZATION OF THE BACTERIOCIN

Cultures of 2 strains (PB1 and RB3) were grown for 6, 12 and 18 h and centrifuged at 10 000×*g* for 10 min at 4°C. The supernatants were stored over 10% chloroform and the cell pellets were washed with 1 M NaCl and the supernatants collected and assayed for bacteriocin activity. The cells were resuspended in a minimum amount of 0,02 ml Tris HCl buffer, pH 8,2, and disintegrated by sonication at an amplitude of 8 microns, using a MSE sonicator giving 3 bursts of 2 min each. During sonication the temperature was kept below 0°C using an alcohol ice bath. Alternatively the cells were disrupted with SDS using the method described by Meynell & Meynell (1970). The disintegrated cell lysate was sterilized with chloroform and assayed for bacteriocin.

5.2.8 THE ISOLATION OF BACTERIOCIN RESISTANT MUTANTS

Overnight cultures of producer strains of *Bacteroides* (PB1, PB2, RB3) were streaked across the surface of agar plates to produce lines or circles of confluent growth, or diluted and spread onto plates so as to produce individual colonies. After 48 h incubation the cells were killed using the chloroform technique described in 5.2.4 and overlayed with 3 ml of soft agar to which 0,2 ml of mutagenised cells had been added. The indicator cells were first treated with NTG as described in 2.2.7. After 48 h incubation the plates were examined and isolated colonies occurring in the zones of inhibition were picked and retested by inoculating onto plates which had been prepared in a similar way to the mutant screening plates but with the indicator cells omitted.

5.2.9 THE EFFECT OF BACTERIOCIN ON RIFAMFICIN RESISTANT MUTANTS OF THE INDICATOR STRAIN (PS1)

Overnight supernatants of the 10 most active producer strains were sterilized with 10% chloroform for 2 h at 4°C and then assayed against rifampicin resistant mutants. The assay plates contained the rifampicin sensitive wild type PS1 cells, or one of 7 rifampicin resistant mutants derived from this strain.

5.3 RESULTS

5.3.1 PATTERNS OF BACTERIOCIN PRODUCTION AND BACTERIOCIN SENSITIVITY

Of the 36 *Bacteroides* strains investigated, 20 were found to produce bacteriocin-like antagonism which inhibited the growth of at least 2 of the other 35 strains they were tested against (Table 5.1). The 36 strains could be divided into 4 groups. Strains in Group A were the strongest bacteriocin producers which had the widest spectrum of activity. The strains in this group were to a large extent resistant to the bacteriocins produced by the other strains, although, all but 2 were sensitive to at least one of the other bacteriocins. Strains in group B all produced some bacteriocin activity but these bacteriocins were only effective against a limited number of strains and in almost all cases only very small zones of inhibition were produced. All the strains in this group were sensitive to a number of bacteriocins produced by other strains. Group C contained 12

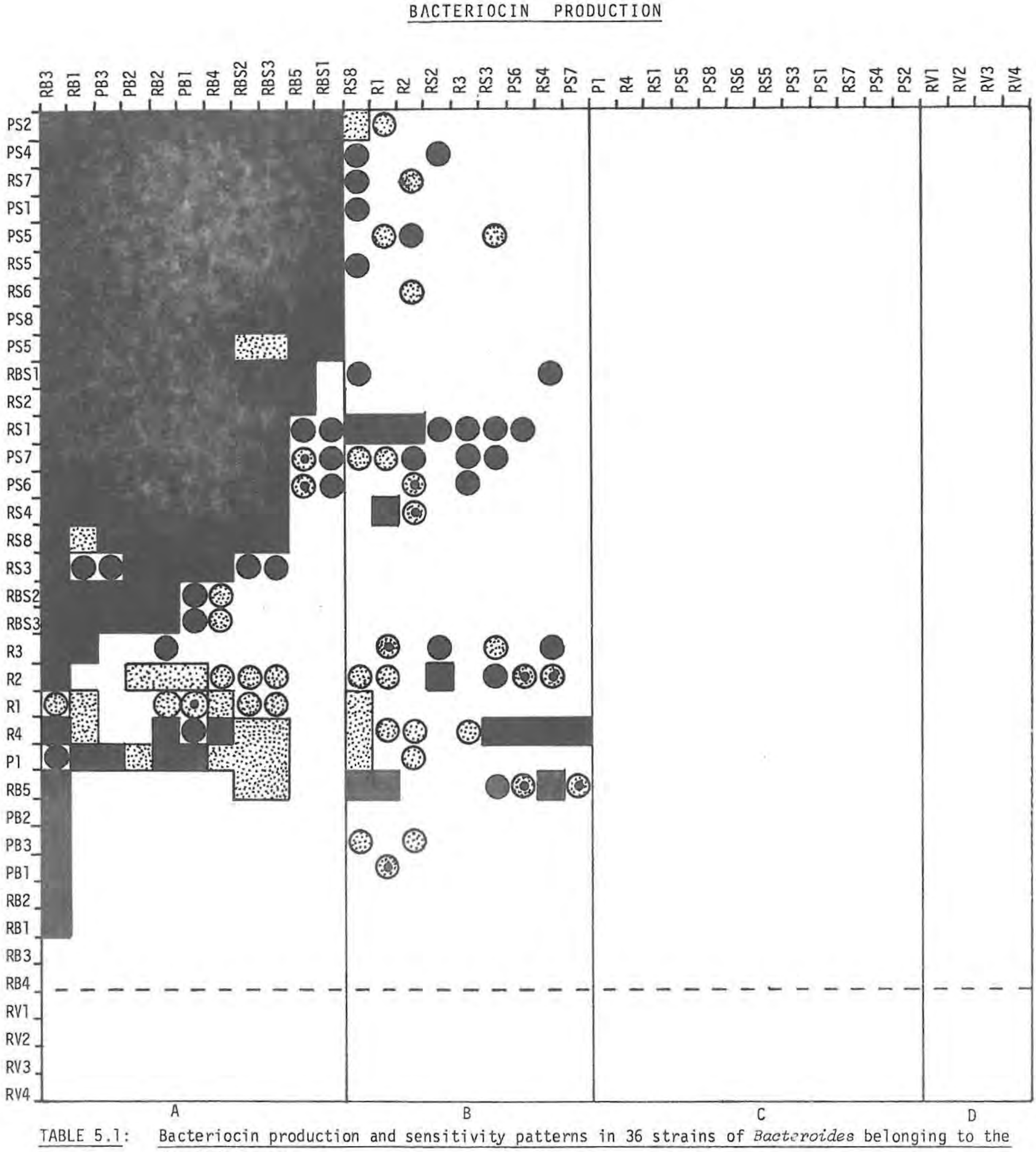


TABLE 5.1: Bacteriocin production and sensitivity patterns in 36 strains of *Bacteroides* belonging to the *fragilis* group.

The strains have been grouped into 4 groups (A,B,C,D) along the horizontal axis according to their ability to produce bacteriocin. On the vertical axis strains have been grouped according to their patterns of resistance to bacteriocin.

KEY:
Black squares - clear zones > 2 mm in width
Black circle - clear zones < 2 mm in width
Stippled square - turbid zones > 2 mm in width
Stippled circle - turbid zones < 2 mm in width
Black centre - small clear zone surrounded by turbid zone.

strains, none of which produced any inhibitory activity against any of the other strains which were tested, however all of the strains in this group showed marked sensitivity to the majority of the bacteriocins produced by the group A strains and most were sensitive to some of the bacteriocins produced by the group B strains. Group D contained only 4 stains and was interesting as these stains were the only 4 stains to show very strong colonial phase variation. All 4 of these stains were resistant to the bacteriocins produced by all the stains tested, and none of them produced any activity against any of the other strains. The significance of the relationship between the 2 phenomena is not known.

The inhibition zones produced by group A strains were normally clear and *c.* 5 mm in width. However on some sensitive strains turbid zones were produced and in some cases only narrow zones < 2 mm in width were produced. Most of the zones produced by the group B strains were quite narrow (< 2 mm in width) and in about half the cases the zones were either turbid or had a very narrow clear zone surrounded by a turbid zone.

Except for the 2 strains RBS 2 and RBS 3 all the other producer strains showed different patterns of activity against the sensitive strains. The patterns of sensitivity exhibited by the various strains also differed in most cases. Again it is interesting to note that the strains RBS 2 and RBS 3 showed similar sensitivity. Both these strains were identified as *B.vulgatus* which suggested that these two strains may have come from the same source. However they were isolated at different times and from sources which were 800 km apart.

None of the 20 producer strains had any inhibitory effect on the 12 species of bacteria belonging to other groups which were tested.

(Fusobacterium varium, Fusobacterium mortiferum, Escherichia coli B, Escherichia coli K12, Shigella boydii, Salmonella typhimurium, Proteus vulgaris, Klebsiella pneumoniae, Enterobacter aerogenes, Serratia marcescens, Clostridium perfringens, Staphylococcus aureus.)

Material isolated from the zones of inhibition of all 20 producer strains was tested for the ability to produce plaques. In no cases were any of the inhibitory effects found to be transmissible.

5.3.2 THE INDUCTION OF BACTERIOCIN

Mit C and UV radiation had little or no effect on the production of bacteriocin in any of the 36 strains tested. Although minor variations in the type and size of some of the smaller zones were noted, these variations were erratic and the results were not reproducible. The patterns of sensitivity remained essentially unchanged under all conditions. Exposure of the 2 producer strains PB1 and RB3 to Mit C and UV radiation had no effect on the amount of bacteriocin produced.

5.3.3 BACTERIOCIN ASSAY

The simplest method for the detection of bacteriocinogeny is the spot test (Mayr-Harting *et al.*, 1972). However this method was not found to be very successful and the well technique described in 5.2.4 was found to be superior in all respects. However even with this technique difusion of the bacteriocin was slow and increased zone sizes were obtained if the plates were held anaerobically overnight at RT or at 4°C before incubation at 37°C.

5.3.4 DETERMINATION OF OPTIMAL CONDITIONS FOR BACTERIOCIN PRODUCTION

The effect of the nutrient status of the culture medium on bacteriocin production was investigated by assaying bacteriocin production in strains PB1 and RB3 grown in MM, peptone yeast extract medium, and BHI broth. No detectable bacteriocin production occurred in MM and bacteriocin production was 2-4 times higher in BHI broth than in peptone yeast extract medium. The same levels of bacteriocin production were obtained after 12 h incubation at 30^{o} and 37^{o}C.

5.3.5 THE RELEASE OF EXTRACELLULAR BACTERIOCIN

In the two strains tested (PB1 and RB3) bacteriocin was first detected in the supernatants of cultures in mid exponential phase and the maximum titres of extracellular bacteriocin were obtained during early stationary phase (Fig. 5.1). In both strains tested a gradual decrease in bacteriocin titre occurred over the remainder of the 72 h period.

5.3.6 THE LOCALISATION OF THE BACTERIOCIN

The greatest bacteriocin activity was found to be extracellular. Washing the cells with 1 M NaCl did not release any significant amounts of bacteriocin indicating that the bacteriocin was not bound to the cell membrane. In addition no significant amounts of bacteriocin were found in the disrupted cell material (Table 5.2).

5.3.7 THE ISOLATION OF BACTERIOCIN RESISTANT MUTANTS

Attempts were made to isolate stable bacteriocin-resistant mutants from 10 different susceptible strains of *Bacteroides* (PS1, PS2, PS4, PS5, PS7, PS8, RBS3, RS1, RS5) following mutagenic treatment with NTG. Resistant colonies were observed to occur in all the strains tested even in the absence of mutagenic treatment. However the number of resistant colonies increased after mutagenic treatment.

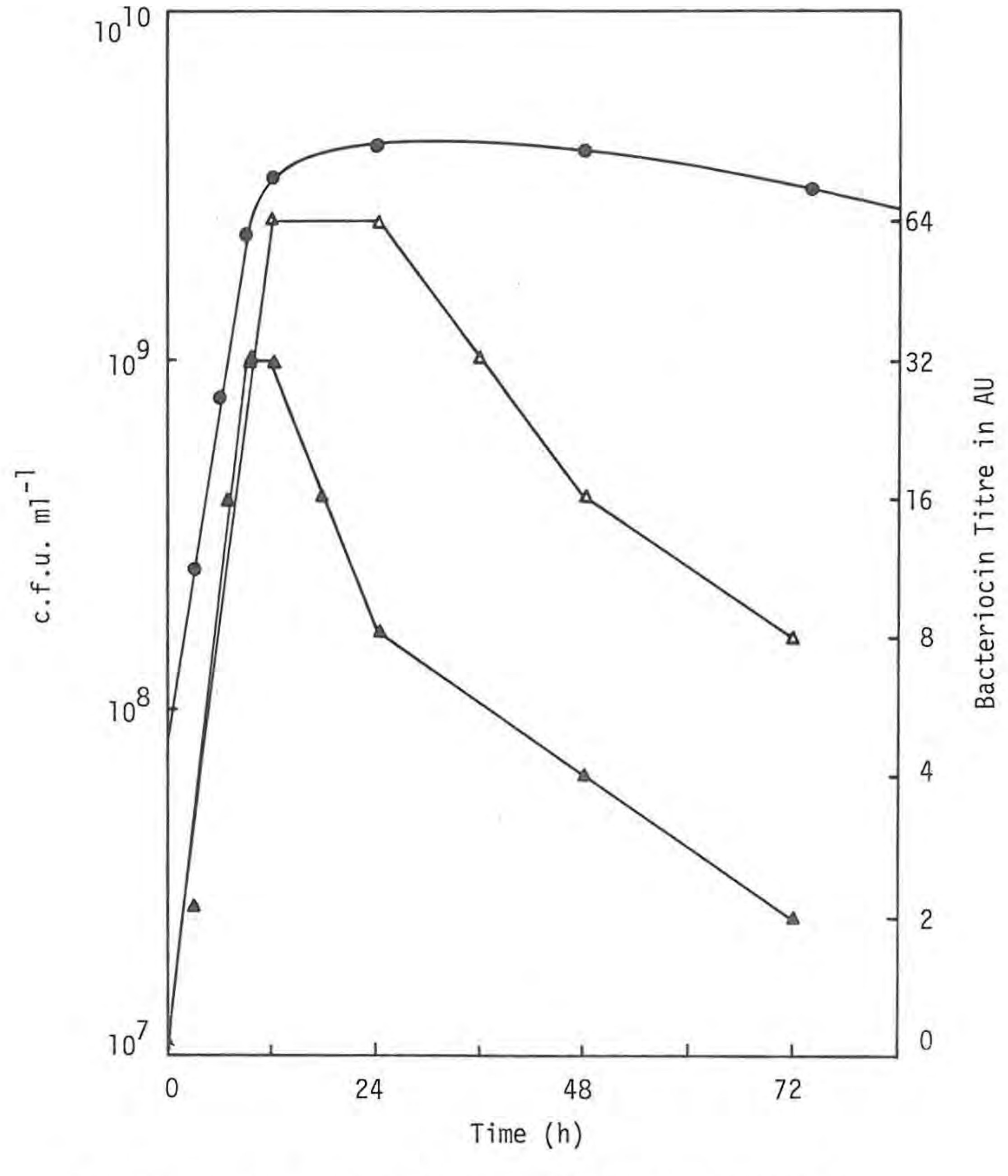


Fig. 5.1. The release of extracellular bacteriocin in *B. fragilis* PB1 and RB2 grown in BHI broth. Growth curve of PB1 and RB2 cells grown in BHI broth (●). Bacteriocin titre in AU produced by PB1 (△) and RB2 (▲).

TABLE 5.2

Localization of the bacteriocin.

Bacteriocin producing strain	Age of culture(h)	Bacteriocin activity (AU)		
		Extracellular[a]	Cell bound[b]	Intracellular[c]
PS1	6	8	2	1
	12	64	2	1
	18	64	2	1
RB2	6	8	2	1
	12	32	2	1
	18	16	2	1

a = Culture supernatant.
b = Cells washed with 1 M NaCl and supernatant assayed.
c = Sonication of SDS treated cells.

A high proportion of these colonies (>50%) showed an increase in opacity and were found to contain encapsulated cells, even in strains which did not normally appear to produce encapsulated cells. Colonies exhibiting altered morphology and containing encapsulated cells were found to produce a high proportion of bacteriocin resistant cells on re-testing. These clones persisted through a number of subcultures but the proportion of colonies containing encapsulated cells decreased rapidly during each subculture until eventually all these variants disappeared from the population. In those strains tested clones containing encapsulated cells were resistant to bacteriophage infection. No stable bacteriocin-resistant mutants were isolated using this technique.

5.3.8 THE EFFECT OF BACTERIOCINS ON RIFAMPICIN - RESISTANT MUTANTS ISOLATED FROM THE INDICATOR STRAIN PS1

An investigation of one of the Bacteriocins (PB1) undertaken by K. Mossie as part of a M.Sc. thesis, showed that this bacteriocin had a novel mode of action. The primary event of the bacteriocin action was found to be the inhibition of RNA synthesis. Since the mode of action of the bacteriocin and rifampicin appeared to be similar, in that both affect RNA synthesis, the action of the bacteriocins of 10 different producer strains were tested against rifampicin-resistant mutants isolated from a susceptible strain (PS1). All the bacteriocins were found to be inactive against between 2 and 6 of the 7 rifampicin resistant mutants (Table 5.3). Those mutants which were susceptible to particular bacteriocins, showed hypersensitivity compared to the wild type strain. Each mutant showed different patterns of resistance and hypersensitivity to the different

TABLE 5.3

The effect of different bacteriocins on rifampicin-resistant mutants isolated from the indicator strain PS1.

Indicator strain PS1	Bacteriocins produced by different strains of *Bacteroides*									
	RS8	RB1	RB2	PB2	PB3	RBS3	RB3	RB4	RBS2	PB1
WT	+	+	+	+	+	+	+	+	+	+
Rif 13	++	++	++	++	++	++	++	++	++	++
Rif 15	++	++	++	++	++	++	++	++	++	-
Rif 1	++	++	+	+	+	+	-	-	-	-
Rif 14	++	++	++	++	++	-	-	-	-	-
Rif 4	++	-	-	-	-	-	-	-	-	-
Rif 2	-	-	-	-	-	-	-	-	-	-
Rif 16	-	-	-	-	-	-	-	-	-	-

- = Not sensitive

\+ = Sensitive

++ = Hypersensitive

bacteriocins (Table 5.3). A number of the bacteriocins showed similar patterns of activity against the rifampicin-resistant mutants These results suggest that all the bacteriocins had a similar mode of action, which appeared to be linked to the target which determines resistance or sensitivity to rifampicin.

5.4 DISCUSSION

Bacteriocins were produced by just over half of the strains of *Bacteroides* tested, and they were found to specifically inhibit other strains of *Bacteroides*. Similar findings were reported by Beerens *et al.* (1966) and Booth *et al.* (1977). Neither the pattern nor amount of bacteriocin produced was significantly altered by exposure to known colicin inducing agents such as UV radiation or mitamycin C. In the 2 bacteriocins investigated, both were found to be released extracellularly into the growth medium at the end of the exponential phase of growth. Both were found to be unstable in the growth medium and maximum titres were present for only a few hours after which a gradual decrease in the titre occurred. The cause of the drop in titre was not investigated.

The partial characterization and mode of action of one of these bacteriocins (produced by strain PB1) has been subsequently undertaken (Mossie *et al.*, 1979). The bacteriocin was found to be proteinaceous and had a low molecular weight (13 500). It was stable between pH 7,5 and 8,2 and the majority of the bacteriocin was found to be thermolabile, but a small proportion (3%) was heat stable. Both temperature and the growth state of the sensitive cells were found to affect killing. The mode of action of the bacteriocin was found to be unusual in that the primary event in its action was the inhibition of RNA synthesis. It was found to inhibit RNA synthesis immediately, followed by the inhibition of protein synthesis after a 20 min delay, presumably as a result of the initial inhibition of RNA synthesis. The bacteriocin had no effect on DNA synthesis.

Since the mode of action of the bacteriocin resembled that of rifampicin, in that both affect RNA synthesis, the action of the bacteriocin was tested on a number of rifampicin-resistant mutants isolated from a sensitive strain (PS1). Of the 10 rifampicin-resistant mutants tested 9 were found to be resistant to the action of the bacteriocin as well. One mutant was found to be hypersensitive to bacteriocin action (Mossie *et al.*, submitted for publication). All the rifampicin-resistant mutants were found to adsorb bacteriocin, and the mutants appeared to be tolerant due to an alteration in the target site of the bacterium rather than in the receptor site. These results suggest a link between the target site of rifampicin and the target site of the bacteriocin action, although these do not appear to be identical, as different mutants showed different degrees of resistance to the bacteriocin. The presence of a bacteriocin inhibitor in crude extracts of the producer strain was suggested, as high concentrations of the bacteriocin inhibited RNA synthesis. The action of bacteriocins from 10 different producer strains were tested against a number of different rifampicin-resistant mutants isolated from a sensitive strain. All of the bacteriocins were inactive against some of the mutants suggesting that they all had a similar mode of action to the bacteriocin produced by the stain PB1. The mutants however showed a variety of patterns of resistance to the different bacteriocins indicating that the variations in the sensitivity of the cell target (RNA polymerase?) to different bacteriocins is highly specific. This complex relationship between the bacteriocin and the cell target, may be responsible for the unique patterns of bacteriocin sensitivity displayed by susceptible strains.

Attempts to isolate bacteriocin-resistant mutants resulted in the isolation of numerous resistant colonies displaying altered morphology and containing a high proportion of encapsulated cells. These resistant clones were not stable and they appeared to have arisen through the selection of encapsulated cell variants in the population which were resistant to the action of the bacteriocin. The exposure of strains which normally did not appear to produce encapsulated cells, to mutagenic agents, resulted in the production of encapsulated clones. Although the bacteriocin partially characterized by Mossie *et al.* (1979) was found to be a protein of low molecular weight it would appear that the presence of a capsule surrounding the cell prevents the action of the bacteriocin, presumably by blocking the receptor sites. The bacteriocin characterized by Booth *et al.* (1977) differed from the one mentioned above in that it appeared to have a much higher molecular weight (> 300 000). The 4 strains of *Bacteroides* which showed strong phase variation were found to exhibit a unique pattern, in that none of them produced bacteriocins effective against any of the strains tested, and none of them were susceptible to any of the bacteriocins produced by any of the other strains. The significance of this observation is not known but it seems unlikely that it is due to coincidence. The results of this investigation indicate that complex mechanisms appear to be present in members of the *B.fragilis* group which can affect the sensitivity of individual strains to the action of bacteriocins. Two different systems appear to be present, one of which acts at the receptor site and one which acts at the target site in the cell.

The properties of the bacteriocins as revealed by laboratory experiments raises a number of interesting questions about the significance of bacteriocins in the natural environment (e.g. the alimentary tract). It might be expected that bacteria which produce bacteriocins would have an advantage over susceptible strains occupying the same ecological niche. Experiments conducted with *E.coli* indicate that there is little,if any, evidence that the ability to produce colicins confers any particular advantage on the producer strain in the alimentary tract (Sears *et al.*, 1950; Robbins *et al.*, 1957; Branche *et al.*, 1964; Vieu, 1964; Cooke *et al.*, 1972). Smith & Huggins (1976) have however, demonstrated that the presence of the Col V plasmid leads to an increase in pathogenicity and the ability to survive in the alimentary tract, but not through the action of the colicin.

In a study of the role of bacteriocin production in strains of *Bacteroides* in the ecology of the colon, Booth *et al.* (1977) observed the simultaneous presence of both bacteriocin producing and susceptible strains in the human colon. Their results showed that not only did susceptible strains persist in the presence of producer strains, but the producer strains remained in the minority (9-35% of the total isolates). Only one producer strain persisted at detectable levels throughout the 25 weeks of the program. In the system studied by these workers the ability to produce bacteriocin did not appear to give the producer strain any advantage over the susceptible strains. They did however point out that due to the complexity of the system, the possibility that bacteriocin production may play some role in the ecology could not be ruled out. The role of bacteriocin production in the ecology of cell complexes growing on the surfaces of the alimentary tract, would be very difficult to determine.

CHAPTER VI

SUMMARY AND CONCLUSION OF PART I

The need for genetic studies on obligate anaerobes has become apparent in recent years. The aim of this study was to establish a genetic system for the controlled mapping of genes in members of the *B.fragilis* group. In addition to the genetic mapping of chromosomal markers, the establishment of such a system could be of value in studies of anaerobic physiology, virulence and the transfer of the determinants of antibiotic resistance in this group. With this end in view a variety of mutants were isolated from a number of strains of *Bacteroides*. During efforts to isolate mutants it became apparent that the mutagenic treatments which were used,caused a rapid loss of viability in the various stains, while low yields of mutants were obtained.

Although the transfer of plasmids by conjugation has been shown to occur in *Bacteroides*, no evidence for plasmid mediated transfer of chromosomal markers has been reported. Burt & Woods (1977) succeeded in obtaining transfection of phage DNA in a strain of *B.thetaiotaomicron*, which resulted in the production of virulent phage particles, however attempts to obtain transformation using similar techniques were unsuccessful. Attempts by the same workers to obtain transduction with the same phage were also unsuccessful. In spite of using a number of different phages and recipients Booth *et al.* (1979) were also unable to obtain transduction in any of the phage-host systems used. In this study attempts to obtain transduction using a number of different phages specific for *B.fragilis* and *B.thetaiotaomicron* also failed to demonstrate transduction. All the phages isolated during this investigation were virulent and attempts to induce lysogenic phages were unsuccessful. Booth *et al.* were also unable to isolate any temperature phages in spite of an extensive search for such phages.

Although there is evidence to show that extrachromosomal DNA can be taken up by *Bacteroides* cells there is no evidence to show that genetic recombination occurs in these cells. In all the bacterial systems which have been investigated so far, chromosomal recombination involving an exogenote has been shown to require a functional recombination system in the recipient cell. This system is also required for the repair of damaged DNA by certain repair pathways, and is essential for the induction of mutation by UV radiation and a number of other mutagenic agents. This system has also been shown to be required for the induction of prophages and a number of other physiological processes in *E.coli*.

The lack of success in obtaining chromosomal recombination in members of the *Bacteroidaceae*, along with the inability to induce prophages, and the difficulties encountered in inducing mutants, pointed to the lack of function of such a system in this group under the conditions used. In the light of this observation, it seemed that an investigation to try and determine whether recombination function was reduced or absent under some or all conditions, would be informative. It was considered that such a study might provide information which could be used to try and induce or optimise recombination processes in these organisms if they were found to be present. Methods which have been developed for the study of bacterial genetics have all been designed for use with aerobic bacteria. Working with anaerobic bacteria presents a number of difficulties and requires many specialized techniques, and it was considered that some of the difficulties experienced in obtaining recombination, mutation and induction in *Bacteroides* might have been due to attempting to apply techniques designed for aerobic bacteria, to studies on anaerobic bacteria.

Experiments using UV irradiation have made significant contributions, both to the study of DNA repair mechanisms, and the induction of mutation. Much of the current information, showing that common enzymatic steps and pathways are involved in the process of recombination, DNA repair and the induction of mutations, has been obtained for such experiments. As the effects of UV irradiation on bacterial DNA are amongst the most extensively studied,the use of this agent appeared to be the obvious choice for such studies on *B.fragilis*. However, before investigating the effect of physical or chemical treatments on the DNA, it is necessary to have some knowledge of the responses of the organism to damage or alterations to the DNA molecule. Thus, a study of the physiological responses of *B.fragilis* to the effects of UV irradiation was considered to be a suitable starting point. The results of this investigation are reported in Part II.

During the course of this investigation a number of interesting features associated with the *B.fragilis* group emerged. A common feature of all the strains studied, was the presence of colonial variation associated with the production of extracellular material by some of the cells within the population . The production of extracellular material appeared to interfere with the attachment of both phages and bacteriocins to receptors on the cell surface and resulted in the cells becoming resistant. Segregation of variants of the opposite cell type at high frequency appeared to be responsible for the establishment of a carrier state in phage infected cells. This phenomenon was, to a large extent, unaffected by growth conditions.

Bacteriocin production was also found to be common among the strains investigated and all those bacteriocins tested were found to be ineffective against a number of rifampicin-resistant mutants isolated from a susceptible strain. This suggested that a common mode of action involving the inhibition of RNA synthesis might occur in all these bacteriocins. A complex relationship between the bacteriocin and the cell target (RNA polymerase?) indicated that this might be the basis of the specific patterns of inhibition observed in this group.

PART II

PHYSIOLOGICAL RESPONSES OF *BACTEROIDES FRAGILIS* TO TO FAR UV RADIATION

CHAPTER VII

THE SURVIVAL OF *B. FRAGILIS* AFTER FAR UV IRRADIATION

7.1 INTRODUCTION

The nature of damage caused by far ultraviolet (UV) radiation and the relationship of such damage and its repair to lethal and mutagenic effects, has been extensively studied in bacteria and their bacteriophages. It should, however, be noted that most of the studies which have been carried out on the physiological responses of bacteria to UV radiation have involved the single species, *E.coli* and its bacteriophages. The vast literature pertaining to the various aspects of UV photobiology has been extensively reviewed and much of the information used in this and the following chapters has been obtained from the following reviews. The nature of photobiological damage has been reviewed by McLaren & Shugar (1964), Smith (1964), Jagger (1967), Wang (1976), Smith (1976), and Smith (1978a). The various physiological and mutagenic responses of bacteria to UV radiation have been reviewed by Rupert & Harm (1966), Doudney (1968), Witkin (1976), and Swenson (1976). The mechanisms of DNA repair have been reviewed by Jagger (1958), Metzger (1964), Hanawalt & Haynes (1967), Setlow (1967a), Setlow (1968), Morsley (1968), Grossman *et al.*, (1975), Hanawalt (1975), Hanawalt & Setlow (1975), Smith (1978b) and Hanawalt, Friedberg & Fox (1978).

7.1.1 FAR UV RADIATION

UV radiation is widely used in the laboratory both as a sterilizing agent and as a tool to study the effects of radiation on biological systems. The UV region of the electromagnetic spectrum is considered to cover the wavelengths between 100 and 380 nm (Jagger, 1967). Both air and water absorb UV radiation below about 190 nm and the region from 100 to 190 nm is therefore called the extreme or vacuum UV region. Atmospheric ozone cuts off solar UV radiation at about 300 nm and this feature is often used to divide the remaining UV region into the near UV region (300 to 380 nm) and the far UV region (190 to 300 nm). However, these definitions are far from universal (Jagger, 1967; Webb, 1977). Nucleic acid bases maximally absorb UV radiation of a wave length of 260 nm, and far UV radiation of this wavelength produces the maximum lethal and mutagenic effects in cells (Kleczkowski, 1951; Jagger, 1967).

In the laboratory the most widely used sources of far UV radiation are low pressure mercury lamps (germicidal lamps) which emit more than 80% of their output at 254 nm which has 85% of the biological activity of the most efficient wavelength, 260 nm (Jagger, 1967). Although the effects of near UV light on biological systems has attracted an increasing amount of attention (Webb, 1977) only the effects of far UV radiation are dealt with in this study, and unless otherwise stated, UV radiation should be taken to mean radiation consisting mainly of the wavelength of 254 nm.

7.1.2 THE NATURE OF DAMAGE CAUSED BY UV RADIATION

DNA has repeatedly been shown to be the primary target for UV radiation in the cell. Long before the biological role of DNA was appreciated and the specific photochemical products in DNA were identified, a number of observations were made which implicated DNA as the major target for UV radiation. It was found that the absorption spectrum of DNA closely follows the action spectrum for both killing and mutagenesis in bacteria. In addition to UV radiation many other agents are known to be responsible for the formation of lesions in the DNA of living cells. These agents may have a direct action in which damage to the DNA is caused by the primary agent, or they may have an indirect action in which the damage to the DNA is brought about by the action of active oxygen species, which are formed by the reaction of the primary agent with a non-DNA target. Far UV radiation acts predominantly or completely by direct action on the DNA, and it is known to bring about a number of different photochemical alterations in the DNA. These are responsible for the adverse effects on cells exposed to UV radiation.

Only pyrimidine and purine residues in the DNA molecule absorb UV radiation. Deoxyribose which makes up about 40% by weight of the DNA, exhibits no absorption at wavelengths above 230 nm and so it does not undergo any direct photochemical alterations (Smith, 1978a). Purines are approximately ten fold more resistant to photochemical alteration than are the pyrimidines (Smith 1978a), and of the different lesions produced by UV, pyrimidine dimers are formed in the greatest quantity and appear to have the greatest biological importance. These cyclobutane type of dimers are formed *in vivo* by lethal and sublethal doses of UV radiation and form between adjacent thymine residues, or, adjacent cytosine residues, and mixed dimers can form between cytosine and thymine residues. The unique chemical stability of the cyclobutane ring formed in dimers of thymine, and the relative ease with which they can be detected, has made this photoproduct the most frequently isolated, assayed and studied. Since their discovery by Beukers & Berends (1960) these photoproducts have played a key role in the design and interpretation of experiments in photobiology. Other photoproducts which are formed in smaller amounts have been studied far less intensively and their biological significance still remains obscure.

Although the number of different types of molecular reactions between pyrimidine residues is limited, the number of possible reactions between pyrimidines and other types of compounds are almost unlimited (Smith, 1978a). Adducts between pyrimidine and other molecules are known to occur in vegetative bacterial cells, but do so in very much smaller numbers than do adducts between two pyrimidines to form dimers. These heteroducts are however, found in very much higher numbers in UV irradiated bacterial spores (Smith, 1978a). The first such heteroduct reaction to be discovered *in vivo* was the cross linkage of pyrimidines and proteins and such heteroducts have been shown to have biological significance (Smith, 1976; Sperling & Havron, 1977). In addition to proteins a number of other compounds have been shown to combine with nucleic acid bases during UV irradiation. Studies on these types of heteroducts are still in their infancy, but it is possible that these types of heteroduct reactions could play a significant role in the radiation biology of the cell.

In addition to the bimolecular reactions discussed above individual pyrimidine residues are also known to undergo a number of unimolecular reactions including hydration, oxidation and reduction after exposure to UV irradiation, but as yet the biological significance of these alterations has not been established (Wang, 1976).

Photochemical reactions involving purines appear to be less important than those involving pyrimidines. Reactions between purine residues subjected to UV radiation do not occur to any significant extent. It has, however, been shown that purines do form heteroducts with other molecules after UV irradiation. They may do so more readily than do pyrimidines (Lenov *et al.*, 1973; Elad, 1976) but as yet the significance of such reactions in the cell has not been established.

In addition to the formation of cyclobutane dimers and other types of adducts, primary chain breaks in the DNA backbone are also found after UV irradiation. These, however, occur in very low yields in contrast to the high yields produced by ionizing radiation and certain photochemical reactions mediated by near UV irradiation in the presence of sensitizers. Although deoxyribose cannot undergo direct photochemical alterations when exposed to UV irradiation, indirect photochemical reactions leading to a small number of breaks in the DNA backbone can occur (Rahn *et al.*, 1974).

7.1.3 THE BIOLOGICAL SIGNIFICANCE OF DAMAGE CAUSED BY ULTRAVIOLET RADIATION

As the bacterial cell contains only one, or at the most a few copies of the chromosome, and due to the vital role of DNA in the cell, damage caused to the DNA has more severe implications for the functional integrity of the cell, than does damage to most other cellular components. The preservation of the unique three dimentional structure of the double stranded DNA helix appears to be a prerequisite for unimpaired function in the cell. Damage to the DNA either by the formation of heterocyclic dimers or by single strand breaks leads to local distortions of the helix. Such distortions may lead to the inhibition of replication and transcription and a deterioration in the fidelity of the DNA.

Both the relatively large size of the chromosome and the chemical nature of DNA make it particularily vunerable to damage, and restoration of the structural integrity of the DNA by repair processes is therefore a vital function of all cells. Three major modes of DNA repair are known; photoreactivation, excision repair and post-replication repair. All three types of repair systems can bring about the repair of cyclobutane type dimers, suggesting the importance for the cell to be able to repair DNA damage under all types of metabolic conditions by means of these three overlapping systems.

One of the first effects of the formation of UV photoproducts is the inhibition of DNA synthesis. For a period following UV irradiation DNA synthesis is almost completely inhibited and then it resumes at an increased rate (Kelner, 1953; Errera, 1954; Hanawalt & Setlow, 1960; Setlow *et al.*, 1963). Kelner (1953) was the first to show that DNA synthesis is much more sensitive to UV radiation than either RNA synthesis or protein synthesis. It was originally thought that the presence of dimers blocked replication and that resumption of DNA synthesis occurred only after the dimers had been removed by repair processes (Setlow & Carrier, 1964; Swenson & Setlow, 1966). Later, Rupp & Howard-Flanders (1968) using excision deficient strains showed that although replication was blocked by dimer formation, DNA was synthesized throughout the chromosome by initiation beyond the dimers. This synthesis between dimers, plus that of repair replication, probably accounts for the small amount of residual synthesis which can be detected, during the period that DNA synthesis is inhibited after exposure to relatively high fluences. During the first 15 min after receiving relatively low fluences of UV giving approximately 35% survival, Billen *et al.*, (1967) showed that 93% of DNA synthesis was semiconservative, and 7% was due to repair replication. The duration of the inhibition of DNA synthesis in *E.coli* has been found to be dependent upon the fluence of UV radiation (Doudney, 1965; Smith, 1969) and the maximum duration of inhibition is 45 min which is equivalent to one division cycle. Doudney (1965) showed that at low fluences the rate of DNA synthesis after resumption was about the same as that of unirradiated controls, but with higher doses the rate of synthesis was reduced.

It has been shown that protein synthesis is necessary for the resumption of DNA synthesis (Doudney, 1959; Harold & Ziporin, 1958; Draculić & Errera, 1959). Doudney (1968) proposed that inhibition of DNA synthesis is due to irreversable damage to the replication site and the resumption of synthesis requires that protein synthesis must occur to bring about reinitiation of DNA synthesis at the chromosome origin. Billen (1969) showed that the original replication point is inactivated after UV irradiation and is not restored by dark repair. Instead new sites of initiation of semiconcervative DNA synthesis appear preferentially at the fixed origin of DNA replication. It has been suggested that in some cases this premature reinitiation at new replication sites could prove lethal to the cell (Billen & Carrier, 1971). Early resumption of DNA synthesis as well as increased viability is obtained if the damaged DNA is repaired by photoreactivation immediately after irradiation (Kelner, 1953; Setlow *et al.*, 1963; Doudney, 1966).

Bridges (1972) has proposed that DNA synthesis is stopped by single strand gaps that arise during repair and that the reinitiation of DNA synthesis after stalling at the replication points, involves a separate dark repair system which is dependent on *rec* A^+ and *uvr*$^+$ gene products. The findings of Radman *et al.*, (1970) have suggested that the inhibition of DNA synthesis may play a role in enhancing repair and increasing survival after UV irradiation by allowing time for excision repair to act in the absence of DNA replication.

Both RNA synthesis and protein synthesis are much less sensitive to the effects of UV irradiation than DNA synthesis (Kelner, 1953). Most of the available evidence indicates that dimers act as blocks which cause the termination of transcription. Evidence from sedimentation experiments (Michalke & Bremer, 1969) has shown that RNA synthesised by irradiated cells is shorter in length than the corresponding RNA synthesised by control cells. As a consequence there also appears to be an increase in the synthesis of abnormally short polypeptide chains in irradiated cells (Brunschede & Bremer, 1969). Wainfan *et al.*, (1963) reported that the synthesis of ribosomal RNA was more sensitive to UV radiation than transfer RNA. Swenson & Setlow (1966) have proposed that RNA synthesis is less affected by UV radiation due to the fact that UV induced lesions occur at sufficiently widely spaced intervals, so that RNA synthesis can take place between the lesions. There are a number of reports (Swenson, 1976) that enzyme synthesis may be inhibited by catabolic repression after irradiation of *E.coli* cells.

7.1.4 THE EFFECTS OF UV RADIATION ON RESPIRATION AND GROWTH

Kelner (1953) was the first to demonstrate that respiration in *E.coli* was partially inhibited after UV irradiation. Later work by Hamkalo & Swenson (1969) and Swenson & Schenley (1970a)showed that UV radiation affected respiration in a unique way. They found that respiration continued at near normal rates for about 60 min after irradiation and then there was partial or complete inhibition lasting for periods up to several hours. The degree of inhibition was found to be dependent on the growth conditions and the fluence of the UV radiation. At high fluences the cessation of respiration was permanent. Hamkalo & Swenson (1969) also observed that the cessation of respiration occurred about the same time that the synthesis of DNA resumed. They proposed that the cessation of respiration occurred as a consequence of DNA damage, and that the slowing down of metabolism takes place so that correction of the unbalanced growth that occurs during the inhibition of DNA synthesis can take place.

Further evidence lead to Swenson & Schenley (1972) proposing a model in which they proposed the presence of a gene, under the control of a repressor, that codes for a protein involved in the control of respiration in the unirradiated cell. They suggested that radiation damage in the cell leads to derepression of the gene and the production of excess protein which causes the permanent cessation of respiration. Inhibition or interference with RNA or protein synthesis after irradiation prevents the inhibition of respiration (Swenson & Schenley, 1970a). Work carried out with repair deficient mutants (Swenson & Schenley, 1974a) has indicated that the induction of the protein responsible for the inhibition of respiration is under the control of the *rec* A and *lex* genes in *E.coli* as are other UV inducible systems.

The immediate biochemical causes of the cessation of respiration are not known but inhibition is associated with the disappearance of NAD and NADP from the cell (Swenson & Schenley, 1970b). In addition cells which have stopped respiring appear to undergo rapid changes in the cell envelope which leads to a marked increase in permeability of the plasma membrane (Swenson & Schenley, 1974b). The destruction of the cell membrane leads to cell lysis which can be detected by a drop of turbidity in the culture. The reason for the rapid deterioration in the integrity of the plasma membrane in cells in which respiration has ceased is not known. In rich media, respiration and growth of unaffected cells may be so rapid that it masks the cessation of respiration occurring in part of the population which has suffered lethal damage.

Swenson's studies on the effects of UV radiation on respiration in *E.coli* have led to him putting forward the intrigueing proposal that radiation death in bacteria occurs as a result of a protein which is induced by radiation damage and is responsible for permanently inhibiting respiration in the lethally damaged cell (Swenson, 1976 & 1978).

Cell growth is fairly closely coupled to respiration in unirradiated and UV irradiated repair-proficient *E.coli* cells (Hamkalo & Swenson, 1969), except where RNA and protein synthesis are inhibited. When respiration ceases or resumes, corresponding growth changes take place, except at high fluences where degradation of DNA appears to lead to complete breakdown in the control processes in the cell.

Under many conditions growth is delayed after UV irradiation. Helmstetter & Pierucci (1968) found that under conditions in which DNA synthesis had been inhibited in essentially all cells, some cell division continued for another 20 min. This division was attributed to cells that had completed the round of DNA replication prior to UV irradiation. This division appears to be responsible for the small increase in viability which occurs immediately after irradiation in cells incubated in liquid medium (Barner & Cohen, 1956; Okagaki, 1960; Smith, 1969; Swenson & Schenley, 1970b). The increase is followed by a plateau or small drop in viability, this is followed later by an exponential increase in viable cells, indicating the resumption of cell division (Smith, 1969; Swenson & Schenley, 1970b; Boyle & Swenson, 1971).

7.1.5 CELL DEATH AND SURVIVAL AFTER UV IRRADIATION

One of the most obvious and important consequences of UV damage to DNA is the death of the cell. Yet in spite of an extensive study of the effects of UV radiation on bacterial cells during the past 50 years the cause of radiation cell death remains poorly understood (Swenson, 1976, 1978). Since the discovery of DNA repair most theories put forward to explain radiation death have involved the disfunction of DNA caused by insufficiencies in repair processes. Contributing to and strongly supporting this point of view is the fact that radiation-sensitive mutants of *E.coli* and other bacteria are almost all deficient in some step or pathway in DNA repair.

There have been numerous explanations put forward to account for the way in which UV radiation causes cell death. The following explanations, which have been put forward by different investigators, are listed in the review by Swenson (1976).

(1) Strand breaks that are formed during excision, accumulate at high fluences because of saturation at the excision step of excision repair (Achey & Billen, 1969).

(2) Dimer excision is inhibited by large UV fluences (Boyle & Setlow, 1970).

(3) At high fluences the gaps in single strands of DNA formed during excision repair begin to overlap more and more and form double stranded breaks (Harm, 1968b; Moss & Davies, 1974; Bonura & Smith, 1975).

(4) DNA replicates prematurely during DNA repair and leads to double stranded breaks in the new duplex of parental and daughter DNA (Hanawalt, 1966).

(5) Abnormal gene dosage and DNA membrane rearrangements arise due to the original replication point not being restored, and due to premature reinitiation at new replication sites (Billen, 1969; Billen & Carrier, 1971).

(6) Abnormalities arise in controlled production of functionally sequenced genome due to dampening of DNA replication over several post-irradiation cycles (Billen & Bruns, 1970 a).

(7) DNA replication is inactivated and not reinitiated (Doudney, 1968 & 1971; Rudé & Doudney, 1973).

(8) Derepression of operons are involved in the formation of unspecified enzymes (Rudé & Alper, 1972).

(9) Derepression of an operon is involved in the formation of a protein which acts as a division inhibitor in filament forming cells (Witkin, 1967).

(10) Cell wall synthesis in filament forming cells does not keep pace with general cell growth which leads to lysis of filaments before division takes place (Brown & Gillies, 1972).

(11) Depression of an operon is involved in the formation of a protein which inhibits respiration (Swenson & Schenley, 1972).

(12) Photochemical formation of DNA-protein cross links occur (Smith *et al.*, 1966).

The survival of cells after UV irradiation may be affected by a number of factors. The level of survival is dependent both on the intrinsic sensitivity of the cellular DNA to photochemical alterations as well as the ability of the cell to repair sublethal damage to the genome. The use of repair deficient mutants has contributed greatly to the understanding of the biological consequences of UV radiation. Studies on the response of bacterial cells to photochemical damage to their DNA have relied heavily on comparisons of cell survival after UV irradiation of repair-proficient and repair-deficient cell types under different pre-and post-irradiation treatments. These types of comparisons rely on accurate plating methods to determine survival curves under different circumstances.

These methods involve dilution of cell suspensions irradiated with various UV fluences and spreading of known volumes onto solid culture media to obtain counts of the number of viable cells. Survival curves obtained by plotting the log of the surviving fraction of colony forming units (c.f.u.), versus fluence, have various shapes which are a consequence of many different factors. Interpretation of the shapes and slopes of survival curves may be made difficult because of the complex nature of events leading to cell death and cell survival.

7.2 MATERIALS AND METHODS

7.2.1 BACTERIAL STRAIN

All experiments conducted on the physiological response of *B. fragilis* to far UV radiation were carried out on a single strain of *B. fragilis* (PS1) which was isolated from a clinical specimen at King Edward VIII hospital, Durban. This particular strain was selected because it had already been used in a number of other investigations and its identity had been confirmed by Dr L.V. Holdman, V.P.I. & S.U. Anaerobe laboratory, Blacksburg, Virginia, U.S.A. This particular strain had been found to be sensitive to all the *B. fragilis* bacteriophages isolated in our laboratory and is sensitive to the great majority of bacteriocins produced by our different strains of *Bacteroides*. In addition a number of different types of mutants have been isolated from this strain.

7.2.2 CULTURE CONDITIONS AND GROWTH PROCEDURES

Cells used in almost all experiments were grown in BHI broth. Stationary phase cultures were obtained by subculturing a loopful of cells from a plate culture into BHI broth and incubating overnight (15 - 18 h) at $37^{\circ}C$. Exponential phase cultures were obtained by diluting different volumes of broth culture into fresh pre-warmed BHI broth under an anaerobic atmosphere (usually a 1 in 10 dilution). The OD of the culture was monitored and the cultures were used when they reached OD giving the required number of cells (OD 0,5 giving *c.* 10^9 cells, ml^{-1}, or OD 0,7 giving *c.* 2×10^9 cells ml^{-1}). Cells that were to undergo irradiation were prepared either by diluting at least 300 times, or by first washing the cells and resuspending to give the required number of cells suitable for irradiation. All solutions and suspending fluids were first checked for the absorbance of different wavelengths of UV light before being used. The most commonly used suspending fluid was Ringer Solution.

7.2.3 UV IRRADIATION

Cell suspensions were normally diluted to give *c.* 10^7 c.f.u. ml^{-1} and 7 ml volumes were placed in flat bottomed glass Petri dishes 9 cm in diameter so as to give a layer of suspended cells of *c.* 0,5 mm in depth. The cell suspensions were shaken continuously during irradiation. The source of UV was a Hanovia portable germicidal lamp fitted with an intergral filter which emitted over 85% of its output at 254 nm. Irradiation was carried out at distances varying between 15 cm and 10 cm below the lamp so as to give an intensity of 0,6 - 0,8 joules m^{-2} s^{-1}. The dose rate was periodically checked with either a YSI - Kettering 65A Radiometer calibrated against an Epply 12 Junction Bismuth Silver Thermopile or a Black Ray UV meter (model J-225, UV Products Inc., California). UV irradiations, dilutions and platings under anaerobic conditions were performed in an anaerobic glove box (Moodie & Woods, 1973b). Aerobic manipulations were carried out on a laboratory bench. Initial work was carried out in a dark room using a red safe light.

7.2.4 MEASUREMENT OF CELL SURVIVAL

Survival curves of cells which had been irradiated with increasing fluences of UV radiation were determined by removing samples at fixed time intervals, and plating them on agar plates. The samples were diluted in Ringer solution and 0,1 ml volumes of the appropriate dilution were spread onto the surface of agar plates which were then incubated under anaerobic conditions for 48 h at 37°C. Each sample was plated onto 4, 5 or 6 agar plates, the number of surviving colonies were counted, and the mean of the results were used to calculate the surviving fraction in each sample. In experiments to determine the survival curves of cells irradiated under anaerobic conditions, both the irradiation procedures and the subsequent dilution and plating procedures were carried out under strict anaerobic conditions in an anaerobic glove box.

7.2.5 ASSAY OF CYCLOBUTYL PYRIMIDINE DIMERS

The method of Setlow & Carrier (1964) was used to assay for the presence of pyrimidine dimers formed during UV irradiation. Some additional modifications of the initial technique (Carrier & Setlow, 1971; Tyrrell, 1973; and Tang & Patrick, 1977a) were incorporated into the method used to assay for dimers in *B. fragilis*. A modification of the method used by Tinnell & Macrina (1976) for the incorporation of labelled nucleotides into the nucleic acids of *B. fragilis*, was used to obtain incorporation of ^{3}H-thymidine into the DNA of *B. fragilis* cells. Pre-warmed tubes of minimal media (Varel & Bryant, 1974), either with or without the addition of 0,2% casamino acids, were inoculated with mid- or late-exponential phase *B. fragilis* cells (OD 0,6 - 0,8). The cells used as the inoculum were either grown in minimal medium or in BHI broth and then centrifuged, washed three times and resuspended in Ringer Solution. The inoculum size was adjusted to give a starting OD of 0,1, the final volume of the culture was 10 ml. These cultures were then incubated at 37^{o}C and as soon as the OD of the culture reached 0,2, ^{3}H-thymidine (Amersham; specific activity 12,8 Ci/mM) was added under anaerobic conditions to give a final concentration of either 20 or 25 μCi ml^{-1}. The cultures were incubated at 37^{o}C and allowed to grow until mid- or late-exponential phase was reached (OD 0,6 - 0,8). The cells were centrifuged at 10 000 x *g* for 10 min at 4°C and washed 3 times and then resuspended in Ringer solution. The cells were diluted to give suspension of either 5 x 10^7 cells ml^{-1} or 1 x 10^8 cells ml^{-1} and the amount of ^{3}H-thymidine which had been incorporated into the cells was measured.

Cell suspensions were irradiated in flat bottomed glass Petri dishes to give survival levels of *c.* 1, 0,5 or 0,1% (fluences of 30, 34 and 38 Joules·m^{-2} respectively) and 0,5 ml volumes of the cell suspension were added to an equal volume of 10% TCA which was held at 0°C for 30 min. All dilutions and manipulations of samples irradiated under anaerobic conditions were carried out in an anaerobic glove box and plate counts were made to determine the number of viable cells in suspension before and after irradiation. Samples were centrifuged at 10 000 x *g* for 10 min at 4°C to separate the acid soluble and insoluble fractions. TCA insoluble fractions were washed once with 5% TCA (0,5 ml) and TCA soluble fractions were extracted 3 times with an equal volume of ether to remove the TCA. Non-radioactive thymine, cytosine and guanine (20 µg of each) was added to each sample to act as markers during chromatography. All samples were then evaporated to dryness in a vacuum desiccator. The dried fractions were then acid hydrolyzed in 0,2 ml of 98% formic acid at 175°C for 30 min. Acid hydrolysis was carried out in sealed thick walled pyrex tubes. After hydrolysis the contents of the tubes were frozen and the pressure which builds up in each tube during hydrolysis was released by holding the tip of the tube in a flame. The tubes were then opened and the hydrolysates were dried in a vacuum desiccator and resuspended in 0,02 ml of 0,01N HCl. This material was then assayed for thymine containing pyrimidine dimers using one- or two-dimentional descending chromatography (Carrier & Setlow, 1971).

The samples were spotted onto 23 x 56 cm sheets of Whatmans No. 1 filter paper and developed by descending chromatography for 16 - 20 h in a solvent consisting of *n* butanol and water, 86:4 (v/v). After the first dimensional chromatograms were dry, the sections containing the thymine dimers were cut out and developed in the second dimension by descending chromatography, for 3 to 6 h using saturated ammonium sulphate, 1 M sodium acetate and isopropanol 80:18:2 (v/v). To determine the percentage of radioactivity in the thymine containing dimers, the thymine region of the first dimension was cut into strips 0,5 cm wide, and these were eluted with 0,5 ml of distilled water for 30 min at 37°C, and the radioactivity was measured using insta-gel (Packard) scintillation fluid in a Beckman scintillation counter.

7.3 RESULTS

7.3.1 THE STABILITY OF THE SOURCE

The output of the UV lamp was checked at 2 to 3 monthly intervals throughout the period of experimentation. A typical calibration curve (Fig. 7.1) shows that the fluence is *c.* inversely proportional to the distance from the lamp (Meynell & Meynell, 1970). The lamp showed a small gradual loss in output during the period of use. In most experiments samples were irradiated at a distance of 10 cm from the lamp which initially gave a fluence of 0,8 Joules m^{-2} s^{-1}.

7.3.2 THE INFLUENCE OF THE SUSPENDING MEDIUM ON THE EFFECT OF UV IRRADIATION

The percentage of UV radiation of wavelength 254 nm which was absorbed by the different buffers used for cell dilution and in liquid holding experiments was measured using a spectrophotometer. The results showed

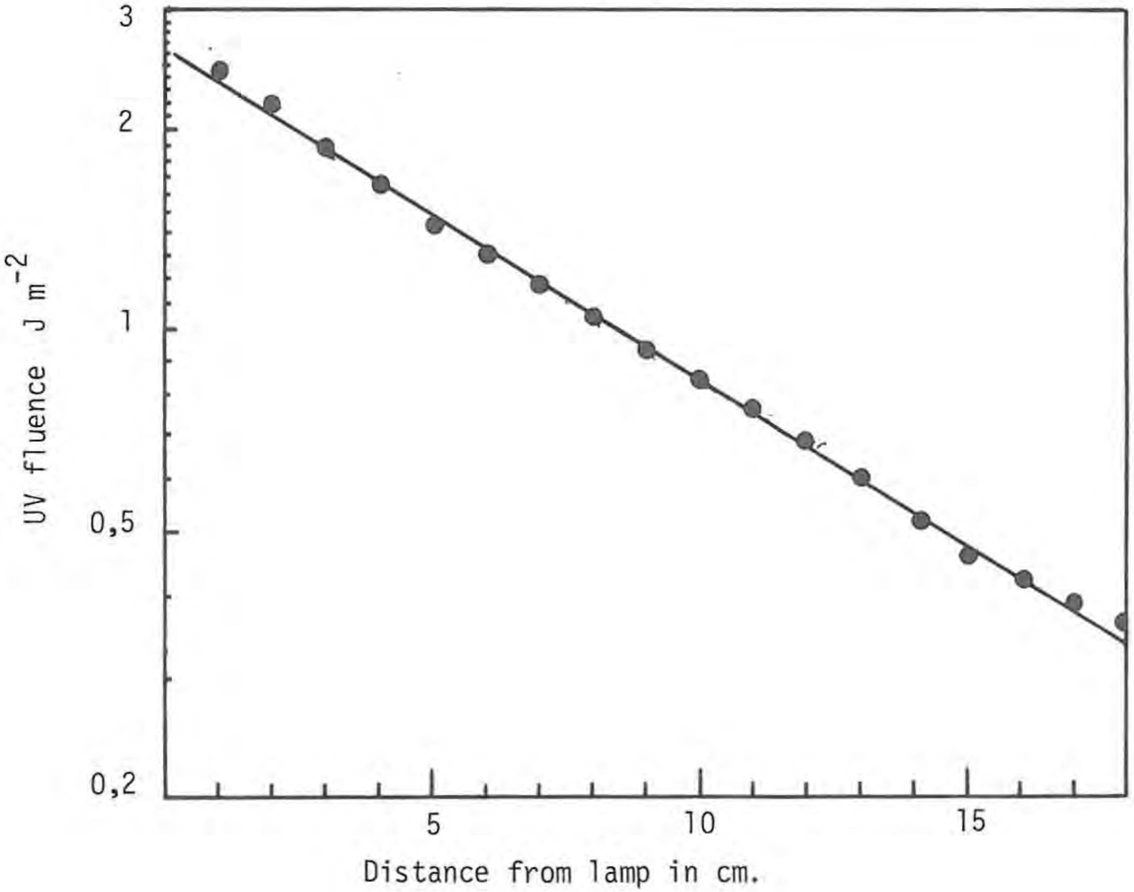


FIG. 7.1 Calibration of UV source

that the reducing agents and indicator used in anaerobic buffers strongly absorbed far-UV radiation (Table 7.1). This necessitated the use of an anaerobic suspending medium which lacked both reducing agents and indicator for anaerobic irradiation procedures. Ringer solution was used as the suspending medium for both anaerobic and aerobic irradiation procedures. However, when Ringer solution was used under anaerobic conditions, it was made anaerobic by first flushing with an anaerobic atmosphere while very hot.

As a standard procedure cells were first centrifuged and washed in buffer twice and then diluted to give cell suspensions of *c*. 10^7 cells ml^{-1} (usually a 1 in 300 dilution). However, for experiments involving the irradiation of cells under anaerobic conditions, it was thought that washing cells under aerobic conditions might affect cell survival after irradiation. A comparison of cell survival with and without washing before diluting 300 times in buffer, was made. Meynell & Meynell (1970) state that a 1 in 300 dilution of a culture in buffer is sufficient to negate any effects of the growth medium in the suspending buffer. The survival curves obtained for both exponential and stationary phase cells indicate that little or no difference in survival was obtained under the two conditions (Fig. 7.2). Similar results were obtained when cell suspensions prepared in the same way were irradiated under anaerobic conditions.

Although cells were normally irradiated while suspended in Ringer solution, the survival of cells which were irradiated on the surface of agar plates was also determined as this procedure was used in one experiment (Fig. 7.3). The results showed that the cells irradiated on the surface of agar plates appear very slightly more sensitive to UV radiation than cells suspended in buffer.

TABLE 7.1

Comparison of different buffers and holding solutions. Percentage of absorbance of UV radiation at 254 nm.

Buffers and holding solutions	% Absorbance
Anaerobe solution (anaerobic)	100%
Minimal salt solution (anaerobic)	100%
Ringer solution (anaerobic)	100%
Ringer solution + sodium thioglycolate	100%
Anaerobe solution (aerobic)	37%
Ringer solution + Resazurin (aerobic)	6%
Minimal salt soltuion (aerobic)	3,5%
Ringer solution (aerobic)	1%
T2 buffer	0,5%

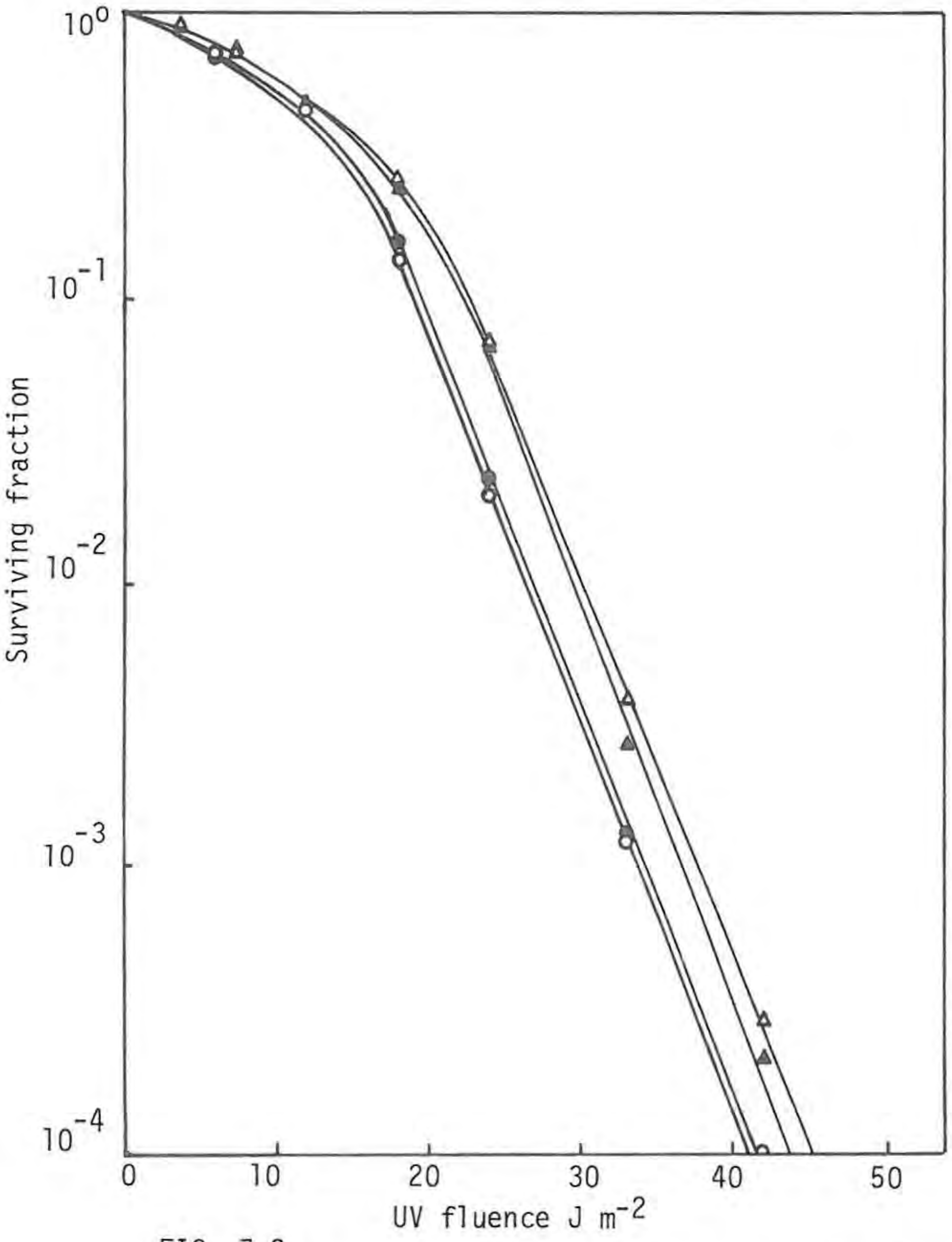


FIG. 7.2

Survival of *B. fragilis* cells irradiated under aerobic conditions after dilution (10^{-7} cells ml^{-1}) with and without washing. Exponential phase cells without washing (o) and with washing (●). Stationary phase cells without washing (Δ) and with washing (▲).

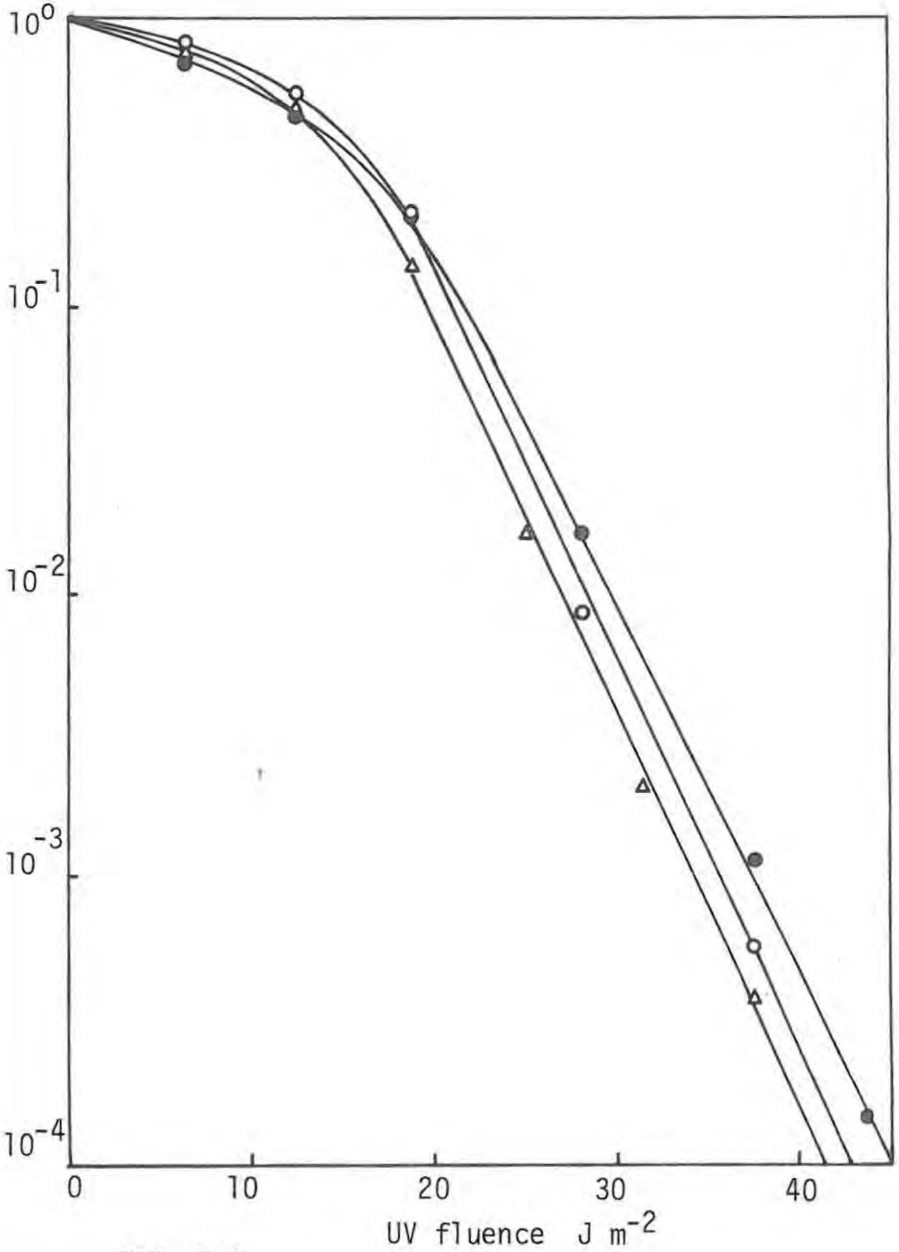


FIG. 7.3

The influence of the type of culture media and irradiation conditions on survival. Exponential cells grown in BHI (o) and MM (●) irradiated under aerobic conditions in buffer. Exponential cells grown in BHI media and irradiated on the surface of an agar plate (Δ).

7.3.3 THE INFLUENCE OF THE GROWTH MEDIUM ON THE SURVIVAL OF CELLS AFTER UV IRRADIATION.

The survival curves for exponential phase cells which had been grown in BHI broth and minimal medium are shown in Fig. 7.3 The cells which had been grown in minimal medium show a slight decrease in sensitivity to UV radiation which is due to a small increase in the size of the shoulder.

7.3.4 THE INFLUENCE OF THE GROWTH PHASE ON CELL SURVIVAL AFTER UV IRRADIATION

The influences of the growth phase on cell survival was determined after irradiation under both aerobic and anaerobic conditions (Fig. 7.4). Exponential phase cell suspensions were prepared from cultures which had been grown to OD 0,7 and stationary phase cell suspensions were prepared from overnight cultures (15 h).

Stationary phase cells show an increased resistance to UV radiation and the difference was particularly marked when the cells were irradiated under anaerobic conditions. The difference between the survival of stationary and exponential phase cells was very much less marked when the cells were irradiated under aerobic conditions, where only a very small increase in the resistance of stationary phase cells occurred. The changes in sensitivity appear to be caused primarily by variations in the shoulders of the survival curves rather than by any marked changes in the final slope of the curves.

7.3.5 THE EFFECT OR IRRADIATING CELLS UNDER ANAEROBIC AND AEROBIC CONDITIONS

A marked decrease in the survival of *B.fragilis* cells irradiated under aerobic conditions was observed when compared with the survival of anaerobically irradiated cells. The extent to which a modification alters the radiation response is called the dose-modification factor (DMF)

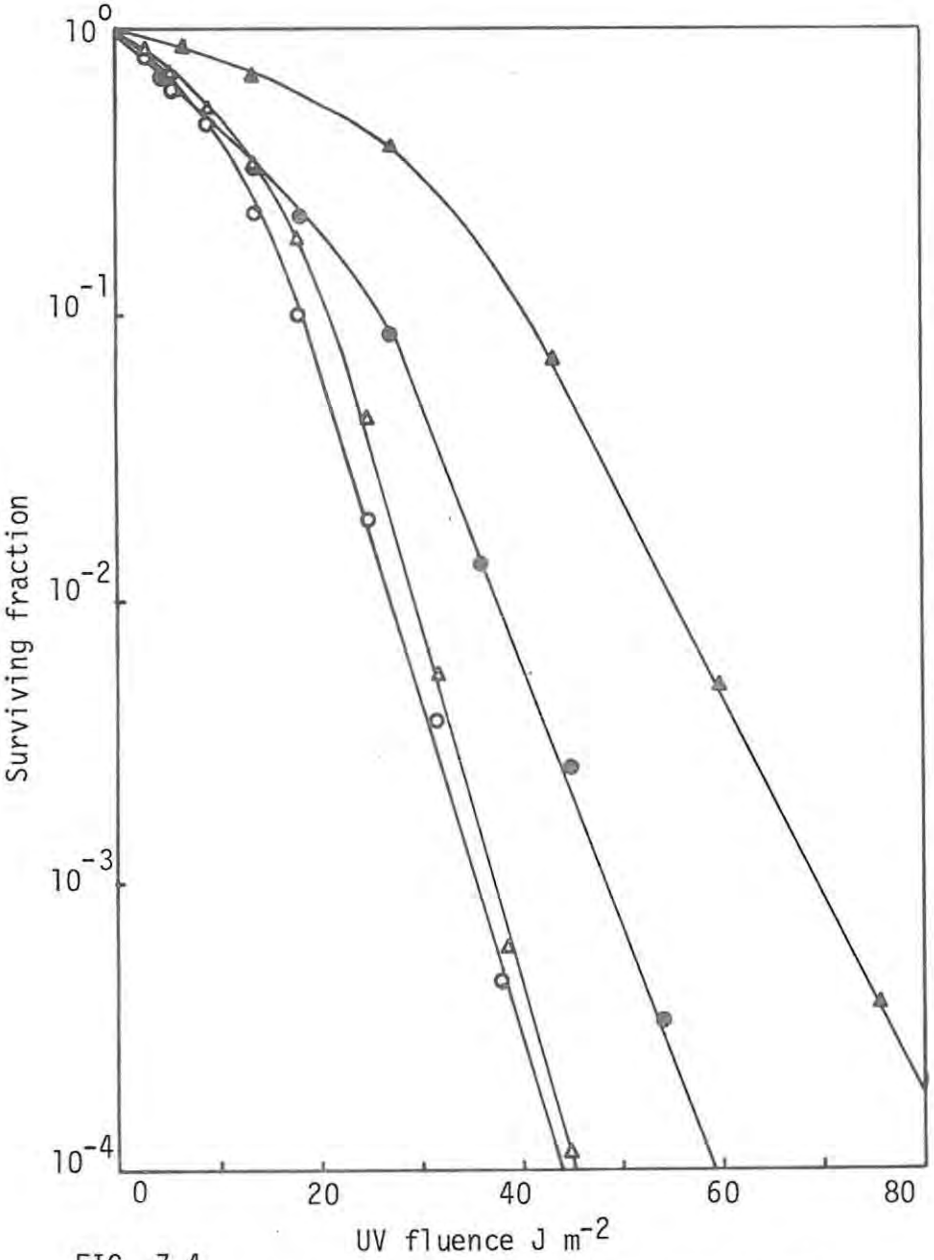


FIG. 7.4

Survival curves of *B. fragilis* cells after UV irradiation under aerobic and anaerobic conditions. Exponential phase cells irradiated aerobically (o) and anaerobically (●). Stationary phase cells irradiated aerobically (△) and anaerobically (▲).

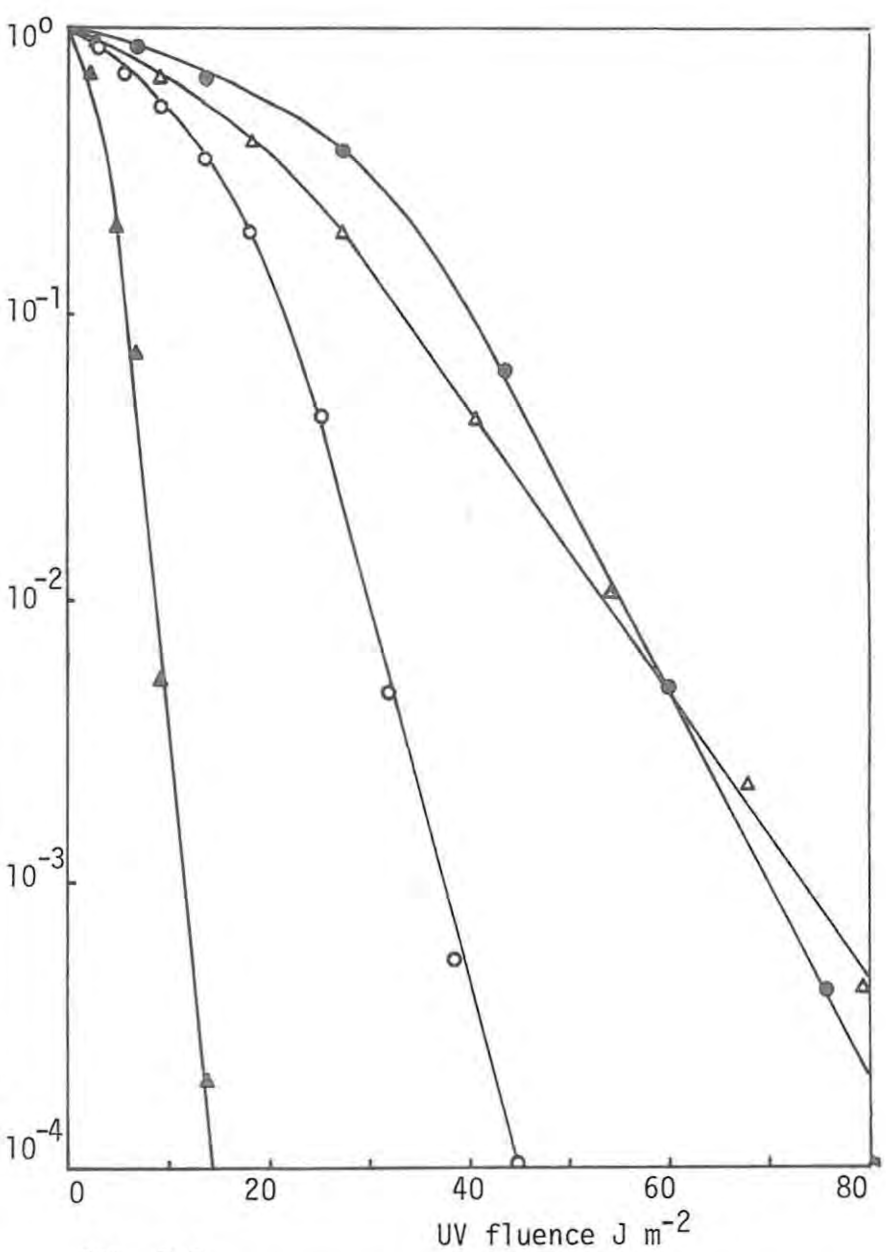


FIG. 7.5

Survival curves of Stationary phase *B. fragilis* and *E.coli* cells after UV irradiation. *B. fragilis* cells irradiated aerobically (o) and anaerobically (●). Aerobically irradiated *E.coli* K-12 wild-type (△) *uvr* A mutant (▲)

$$\text{DMF} = \frac{\text{Dose for a given effect}}{\text{Dose for the same effect in presence of the modifier}}$$

If the modifier decreases the effect of the irradiation (DMF<1) it is called the dose-reduction factor (DRF), and if it increases the radiation effect (DMF>1) it is called the dose-enhancement factor (DEF). In stationary phase cells the DEF due to the presence of air was 1,87 at 1% survival and in exponential phase cells it was 1,41. The increase in sensitivity was mainly due to a decrease in the size of the shoulder rather than any major change in the final slope of the curves.

7.3.6 A COMPARISON OF THE SENSITIVITY OF *E.COLI* AND *B.FRAGILIS* CELLS TO UV IRRADIATION

Stationary phase (15 h) repair competent *E.coli* K-12 cells, and a repair deficient *E.coli* K-12 mutant (*uvr* A^-) were irradiated under aerobic conditions, and the survival curves plotted (Fig. 7.5). The survival curves obtained in this study were similar to those published for these strains by other workers (Moss & Davies, 1974).

A comparison of the stationary phase survival curves for the *E.coli* strains and *B.fragilis* showed that when *B.fragilis* cells were irradiated under anaerobic conditions survival was quite similar to the survival of repair competent *E.coli* cells (Fig. 7.5). However, when *B.fragilis* cells were irradiated under aerobic conditions a marked increase in sensitivity was observed. Survival levels were midway between those obtained for repair-proficient and repair-deficient strains of *E.coli*.

7.3.7 THE EFFECT OF REPAIR INHIBITORS ON THE SURVIVAL OF IRRADIATED *B.FRAGILIS* CELLS.

B.fragilis differs from the other groups of bacteria which have been investigated so far, in that sensitivity to UV radiation varies, dependent on whether irradiation was carried out under aerobic or anaerobic conditions. Experiments were carried out to try and establish whether the different levels of survival were due to differences in the amount of damage to the DNA, or due to differences in the amount of repair occurring after irradiation. Inhibitors known to inhibit different repair pathways in *E.coli* were incorporated into the plating medium used to determine the fraction of c.f.u. surviving after irradiation, in an attempt to inhibit possible repair processes.

Caffeine which is known to inhibit excision repair processes and thus inhibit both liquid holding recovery and host cell reactivation in *E.coli* (Swenson, 1976; Fong & Bockrath, 1979), was incorporated into the plating medium (BHI agar). The m.i.c. for caffeine incorporated into agar plates was determined as 2,5 mg ml^{-1} and a concentration of 1 mg ml^{-1} which was slightly lower than the m.i.c. used. When cells were plated onto caffeine plates after irradiation under both anaerobic and aerobic conditions a marked decrease in the number of surviving c.f.u. occurred (Fig. 7.6). The difference in the survival curves obtained was due to a very marked reduction in the size of the shoulder while the final slope of the curves remained unchanged. The reduction in the size of the shoulder occurred both in cells irradiated under anaerobic conditions and aerobic conditions so that the shapes of the two survival curves obtained on caffeine plates were very similar. A small shoulder remained and in different experiments the size of this shoulder varied to some extent (see similar curves in Figs. 8.12, 10.4 & 10.5).

In addition to caffeine, sodium arsenite was also incorporated into the plating medium as it is thought to inhibit *rec* A dependent steps in DNA repair in *E.coli* (Rossman *et al.*, 1975). The m.i.c. of sodium

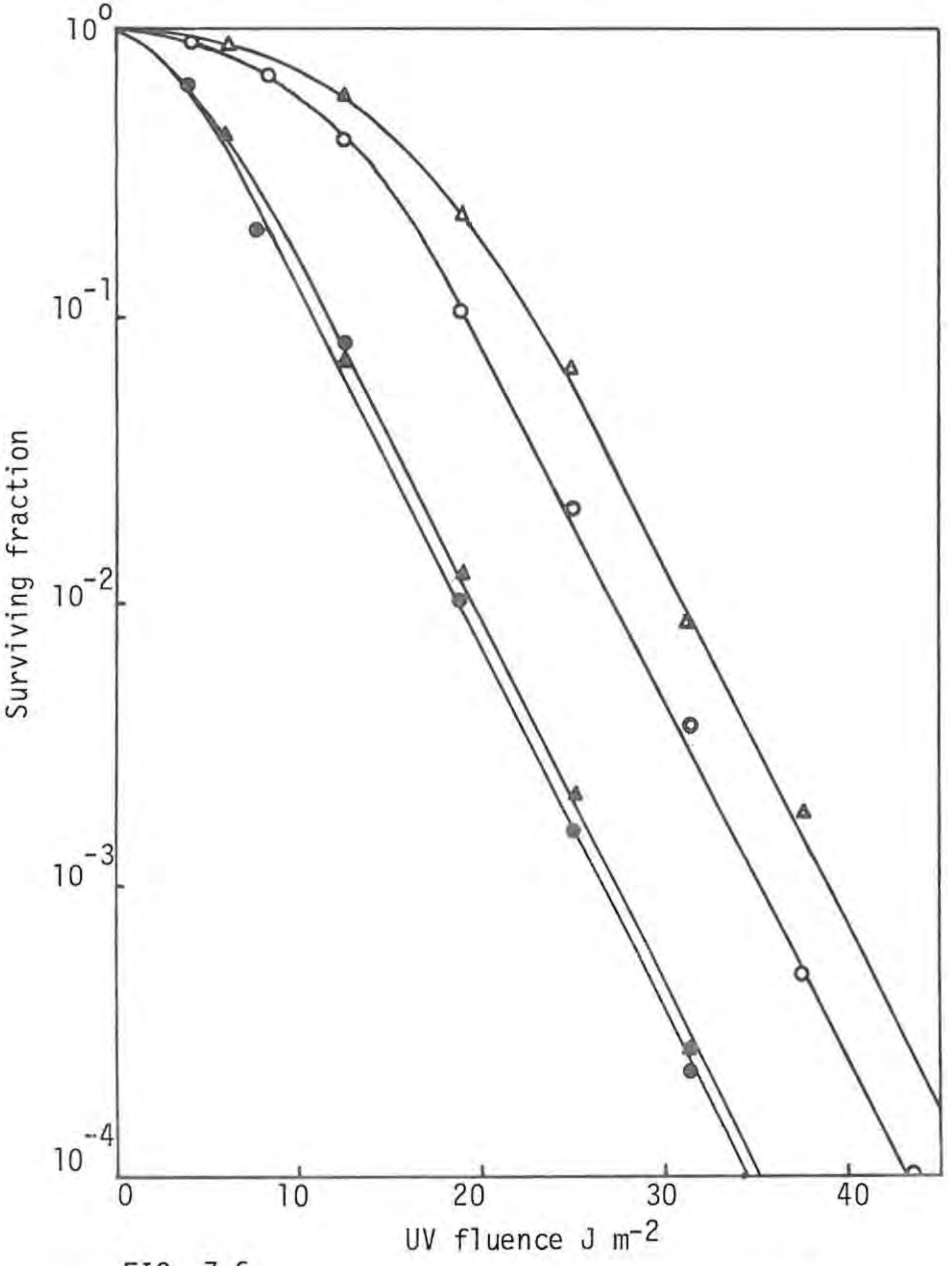


FIG. 7.6

Survival curves of exponential phase *B. fragilis* cells irradiated aerobically and plated onto BHI plates (o) and caffeine plates (●) and irradiated anaerobically and plated onto BHI plates (Δ) and caffeine plates (▲).

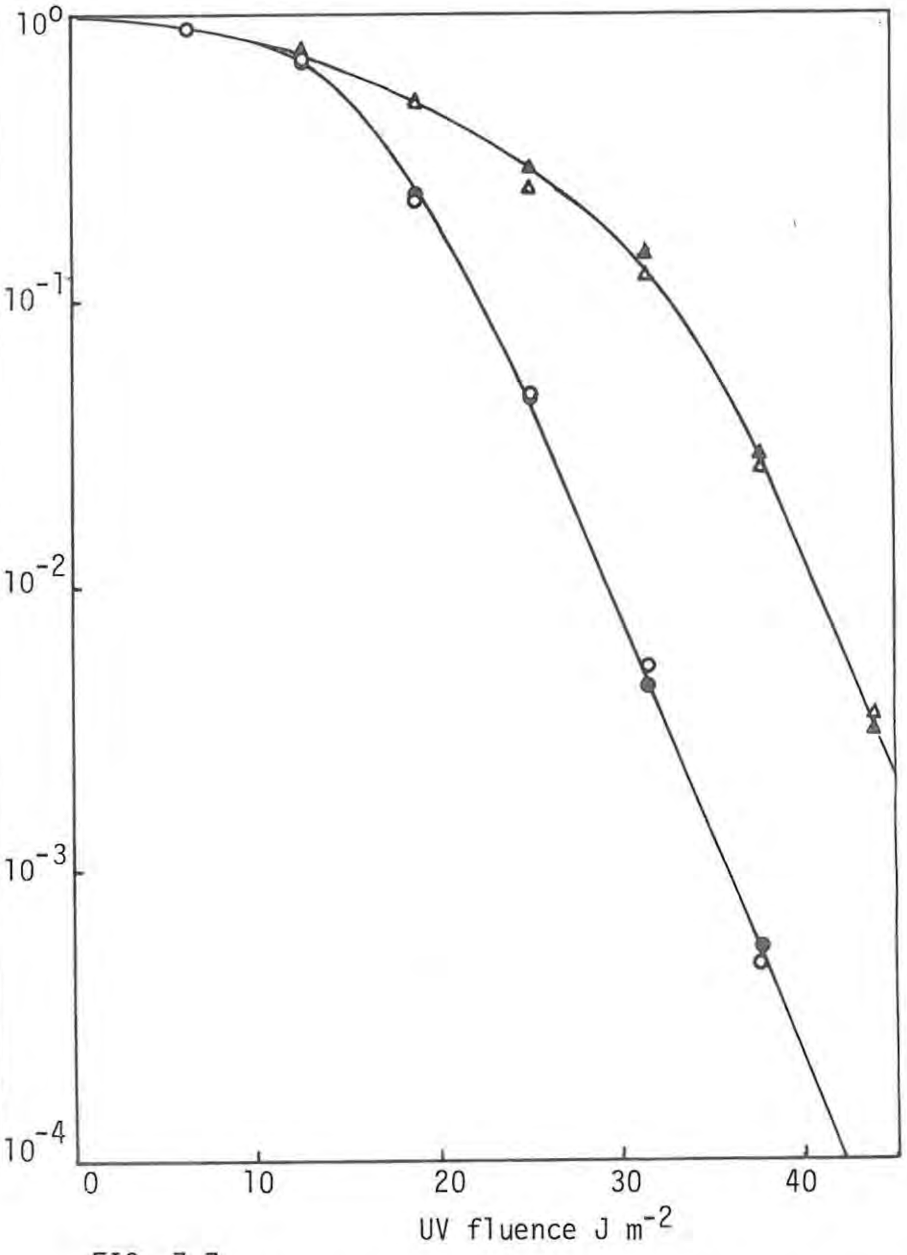


FIG. 7.7

Survival curves of exponential phase *B. fragilis* cells irradiated aerobically and plated onto BHI plates (o) and sodium arsenite plates (●), and irradiated anaerobically and plated onto BHI plates (Δ) and sodium arsenite plates (▲).

arsenite in agar plates was determined to be 130 µg ml^{-1} and a concentration just lower than the m.i.c., which had no effect on the survival of unirradiated cells, was used in the plating medium (100 µg ml^{-1}). From the survival curves obtained after plating on BHI plates containing sodium arsenite, it can be seen that this inhibitor did not affect the survival of cells irradiated under anaerobic or aerobic conditions (Fig. 7.7).

7.3.8 THYMINE DIMERS FORMED DURING IRRADIATION UNDER ANAEROBIC AND AEROBIC CONDITIONS

In an attempt to establish whether a difference in the intrinsic sensitivity of DNA contributed to the different levels of survival after irradiation under anaerobic and aerobic conditions, the number of thymine dimers present in the DNA of the irradiated cells was assayed. The DNA of unirradiated cells was assayed as a control.

Four separate experiments were attempted. Some difficulty was encountered in obtaining sufficiently high levels of incorporation of ^{3}H-thymine into the DNA. Radioactive counts of between 20 000 and 35 000 c.p.m. were obtained for both acid soluble and insoluble fractions and between 0,03% and 0,08% of the ^{3}H-thymine occurred as dimers in irradiated samples (Fig. 7.8). Attempts to obtain higher levels of incorporation in order to ensure greater accuracy of the results, lead to smearing of the labelled nucleotides in the chromatograms with a concomitant loss of definition. In the chromatograms the location of the markers and labelled regions was well defined and the R_f values calculated for the various spots were in agreement with the published values of Carrier & Setlow (1971).

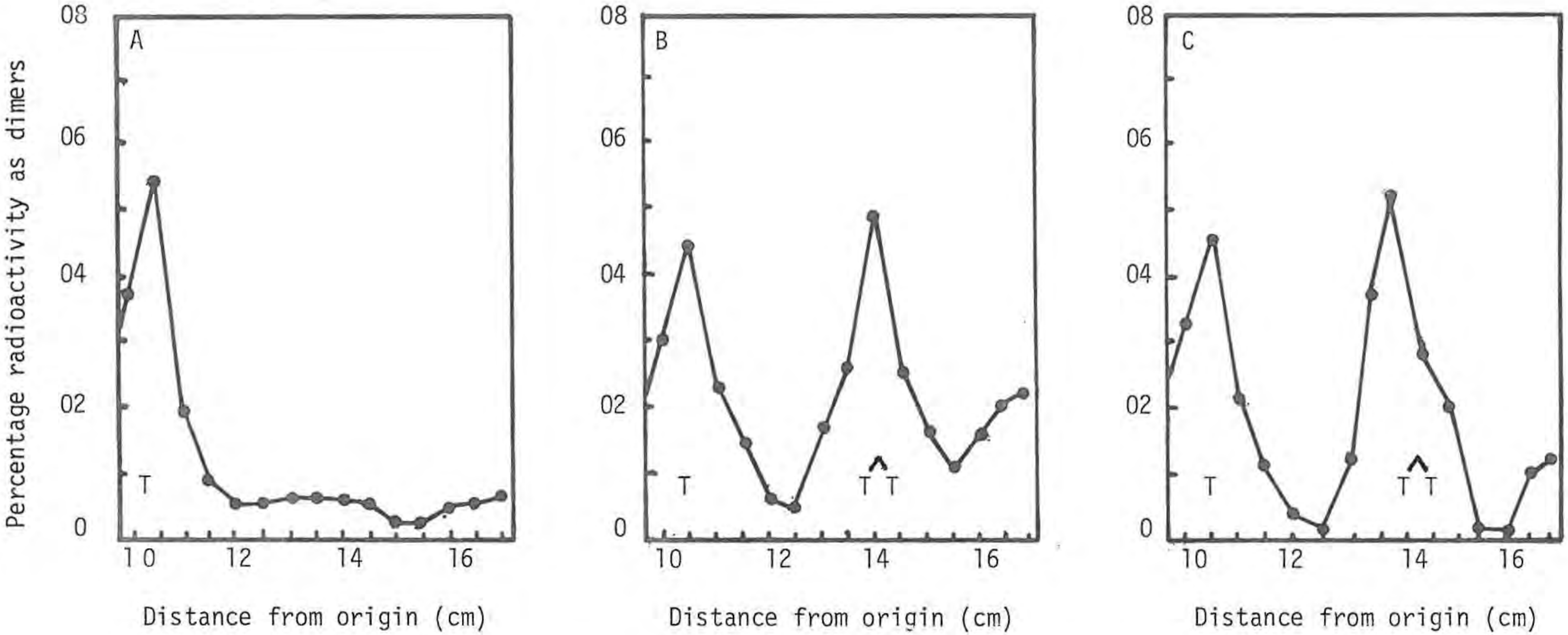


FIG. 7.8 The percentage of thymine-containing pyrimidine dimers induced in the DNA of *B.fragilis* (acid insoluble fractions) in (A) unirradiated cells, (B) cells irradiated under anaerobic conditions (20 Joules m^{-2}), (C) cells irradiated under aerobic conditions (20 Joules m^{-2}).

(T) - Thymine
($\widehat{T\ T}$) - Thymine dimers
The ($\widehat{T\ T}$) markers peaked between 12,5 - 15,5 cm corresponding to an Rf value of 6,4.

The percentage radioactivity in the dimer region from the acid insoluble fractions are shown in Fig. 7.8. The results indicate that the number of dimers produced when cells were irradiated with the same fluence under anaerobic and aerobic conditions were very similar.

7.4 DISCUSSION

The survival curves obtained by plotting the log of the surviving fraction of c.f.u. versus the fluences may have various shapes. In *B. fragilis* as well as in many other repair competent bacterial cells, the survival curves are characterized by the presence of a shoulder which has nearly zero slope at low fluences of UV radiation and a steeper slope (more negative) which tends to be exponential at higher fluences. Many of the factors which bring about changes in the sensitivity of the cells, do so by causing increases or decreases in the size of the shoulder of surviving cells rather than effecting the slope of the final part of the curve.

Haynes (1966) was the first to propose that the increase in cell death which marked the transition from the shoulder to the exponential part of the curve, was caused by a decrease in the efficiency of repair of the photochemical damage in the DNA. Haynes *et al.*, (1968) proposed that the position of each point on the survival curve is a function of both the initial damage (lethal hits) plus the degree to which these hits are repaired. Since then a number of explanations have been put forward to account for the existance of the shoulder on UV survival curves. Almost all of these models regard the shoulder region as one in which the most efficient repair takes place (Swenson, 1976). It has been suggested that at high UV fluences repair enzymes become saturated due to the increased number of lesions, or that the repair enzymes themselves become inactivated directly, by high UV doses (Haynes, 1966). The model which appears to provide the best explanation is the one put forward by Harm (1968b) and later extended by Moss & Davies (1974). They suggested that the repairable regions on the complementary strands begin to overlap as the fluence increases, which prevents the formation of complete copies of the genetic material in these regions. They suggest that the amount of damage that can be repaired by the excision repair mechanism is related to the proximity of the UV-induced lesions to one another, along the DNA helix. It is known that in excision repair a length of nucleotides is removed during the repair process and replaced by new bases using the complementary strand as the template. As the density of lesions along the helix increases with the increasing UV dose there would be a progressively greater probability that excised regions on complementary strands would overlap resulting in the production of irrepairable lesions, loss of the template, and possibly strand breaks in the chromosome. Available evidence strongly supports the explanation that the shoulder region of the UV survival curve represents increased survival levels in cells due to efficient repair which takes place after exposure to low fluences of UV radiation. This suggests that conditions under which an extended shoulder occurs are conditions under which repair is able to occur most efficiently, and where a reduction in the size of the shoulder occurs this is probably due to a decrease in the efficiency of repair.

The survival of cells can be influenced by the conditions to which cells have been subjected, before irradiation. The growth phase of the cells at the time of irradiation has been found to be particularly important in this respect (Swenson, 1976). The nutrient status of the cells at the time of irradiation can also have a marked effect on their later survival (Swenson, 1976). Both these factors appear to be related to the time taken for DNA replication to resume after UV irradiation, and there is good evidence to suggest that under conditions where the resumption of DNA synthesis is delayed after UV irradiation, excision repair is able to operate more efficiently, leading to increased survival (Radman *et al.*, 1970).

Although the type of growth medium in which *B. fragilis* was grown before irradiation appears to have little effect on cell survival after irradiation, the slight increase in survival that was obtained in cells grown in minimal medium could be interpreted as being due to the fact that cells grown in minimal medium might take slightly longer before DNA replication begins, after plating out the cells on BHI agar, thus allowing more time for repair to take place.

It has been reported on numerous occasions that repair proficient strains of *E.coli* show greater sensitivity to UV radiation when in exponential growth than in stationary phase (Durham & Wyss, 1956; Ginsberg & Jagger, 1965; Hanawalt, 1966; Morton & Haynes, 1969; Tyrrell *et al.*, 1972). The difference in survival is due to the decreased size of the shoulder in survival curves of exponential phase cells, while the final slopes of survival curves tend to be similar for the two growth phases. The increase in sensitivity in exponential growth is associated with the excision repair capabilities of the cells (Hanawalt, 1966; Morton & Haynes, 1969; Tyrrell *et al.*, 1972). Strains capable of excision repair (including *rec*-

strains of *E.coli*) show substantially increased sensitivity during exponential growth (Tyrrell *et al.*, 1972). However, strains deficient in excision repair (*uvr*$^-$ strains) did not show an increase in sensitivity during exponential growth. Hanawalt (1966) suggested that the cause of the sensitivity of repair proficient strains in exponential growth was the onset of DNA replication that takes place after the excision step has occurred but before the completion of the polymerization and rejoining steps have occurred. Attempted replication of the resulting single stranded regions of DNA would result in lethal configurations. The survival curve obtained for *B. fragilis* after exponential phase and stationary phase cells were exposed to UV radiation, also showed a marked decrease in the survival of exponential phase cells. This decrease in survival was largely due to a decrease in the size of the shoulder. However, in the case of *B. fragilis* cells, this increase in sensitivity was also linked to whether the cells had been irradiated under anaerobic or aerobic conditions.

Inactivation of bacteria by far UV light has repeatedly been shown to be independent of the presence of oxygen in all organisms which have been studied so far (Zetterberg, 1964; Webb & Lorenze, 1970; Webb, 1977). This is the opposite of the effect of oxygen on near UV light where a marked lethal effect occurs (Webb, 1977). This fact has been used to distinguish between the biological effects of far UV and near UV radiation. In *B.fragilis* the marked differences in survival encountered when cells were irradiated under anaerobic or aerobic conditions suggests that this obligate anaerobe differs from other bacteria which have been investigated, in that the lethal effects of far UV radiation are partially oxygen dependent. The difference in survival curves obtained for *B.fragilis* cells irradiated under anaerobic and aerobic conditions is also largely due to differences in the size of the shoulder, rather than to any marked changes in the final slope of the curve. This suggests that the very marked increase in the sensitivity of the cells irradiated under aerobic conditions might be due to a decrease in the efficiency of repair processes, rather than an increase in the actual photochemical damage to the DNA under aerobic conditions.

Further experiments carried out to investigate these possibilities have tended to confirm this suggestion. The results obtained from the dimer assays indicated that the number of cyclobutane type dimers formed after irradiation under anaerobic and aerobic conditions are not markedly different, and it does not appear that differences in the amount of photoproducts formed can account for the difference in survival that has been observed. Caffeine has been shown to inhibit liquid holding recovery in *B. fragilis* (3.3.6), and there is evidence to suggest that its action is the same as in *E.coli* and other bacteria, where it acts to block excision repair (Fong & Bockrath, 1979). *B. fragilis* cells which were plated onto caffeine plates immediately after irradiation show a marked drop in survival which is reflected as a decrease in the shoulder. The results obtained from the caffeine plating experiments suggest that both the increase in survival of stationary phase cells and in the cells irradiated under anaerobic conditions were due to excision repair processes which were almost completely inhibited in the presence of caffeine. This does not exclude the possibility that other repair systems contribute in a small part to repair or are involved in some other way. The lack of any effect on the survival of cells plated onto BHI agar containing sodium arsenite is interesting and can be interpreted as either that no *rec* A mediated repair processes are involved in the immediate post-irradiation repair of DNA damage, or that if repair processes involving an

E.coli type *rec* A gene product do occur in *B. fragilis* they are not inhibited by the presence of sodium arsenite. It would seem therefore that an excision repair system is responsible for the shoulder which occurs in the survival curve of *B. fragilis* cells after UV irradiation, and that excision repair is able to occur more efficiently in stationary phase cells. This repair process differs from other systems which have been studied, in that although some repair occurs after irradiation under aerobic conditions the level of repair is much higher after irradiation has occurred under anaerobic conditions. It is not clear whether the difference in the level of repair observed is due to the reduction in the efficiency of a single system when exposed to oxygen, or whether 2 different systems or components are involved, one being inhibited in the presence of oxygen and the other functioning independently of oxygen.

CHAPTER VIII

THE RECOVERY OF *B. FRAGILIS* CELLS AFTER UV IRRADIATION

8.1 INTRODUCTION

The recovery of bacterial cells following UV irradiation can be influenced by many factors, and a variety of pre- and post-irradiation treatments have been used in the laboratory to study the way in which these different factors are able to affect cell recovery (Rupert & Harm, 1966; Swenson, 1976). Many of the treatments used have been found to affect recovery by retarding growth and DNA synthesis in irradiated cells, and allowing repair processes to operate more efficiently. The use of inhibitors, especially those which inhibit repair processes, have also proved to be extremely valuable in these studies.

Many of the original studies investigating the UV death and recovery of repair competent cells, used *E.coli* B cells and their derivatives. In many of the later studies, *E.coli* K-12 strains replaced *E.coli* B strains as the experimental organism. *E.coli* K-12 strains belong to a sexual line which offers ease of genetic manipulation in constructing special multiple mutants. Many pre- and post-irradiation treatments which enhance recovery after UV irradiation were discovered using an *E.coli* B strain. These treatments are much more effective in this strain than in either the radiation resistant B/r strain which was developed from it, or in the *E.coli* K-12 strains.

8.1.1 FILAMENT FORMATION

Both *E.coli* B and B/r are repair competent but the latter is more resistant to radiation (Witkin, 1946). The difference in survival after UV irradiation between the two strains is related to the ability of strain B to form long nonseptate filaments, when incubated in a growth medium or other agents that inhibit DNA synthesis (Witkin, 1947; Errera, 1954). Very low fluences of UV radiation, that have only slight effects on survival will cause filament formation in nearly every cell of an *E.coli* B culture (Deering & Setlow, 1957). Filament formation is genetically controlled and the phenotypes of *E.coli* B and B/r cells are sometimes designated *fil*$^+$ and *fil*$^-$ respectively. A radiation sensitive K-12 mutant, *lon*, that formed filaments after exposure to UV or ionizing radiation was isolated by Howard-Flanders *et al.* (1964b). These mutants formed watery mucoid colonies when plated onto rich solid media. The *lon* gene has been shown to be identical to the *cap* R gene which regulates mucopolysaccharide synthesis (Markovitz, 1964; Markovitz & Baker, 1967; Zehnbauer & Markovitz, 1978). Mutants in the *lon* (*cap* R) gene are pleiotrophic and they form nonseptate filaments after UV or nitrofurantoin treatment. In the absence of such treatment the synthesis of the ten enzymes involved in capsular polysaccharide (colanic acid) production are derepressed and an overproduction of the polysaccharide occurs, resulting in mucoid colonies. Zehnbauer & Markovitz (1978) suggested that the *lon* gene product acts as a repressor, and they have cloned the gene in an attempt to identify and isolate the *lon* gene product. Double *lon uvr* strains are much more sensitive to UV radiation than strains with either single mutations, and the fact that *lon* mutants can carry out excision repair shows that the mutants are radiation sensitive for different reasons (Howard-Flanders *et al.*,1964a). The genes from *E.coli* have been transduced into wild-type K-12 strains and the radiation sensitive colonies selected are *lon* and mucoid (Donch & Greenberg, 1968; Donch *et al.*, 1969). The non-filamentous character of B/r cells is due to a mutation (*sul*) which suppresses the *lon* gene (Donch *et al.*, 1969). The *lon* mutants are deficient in the ability to carry out cross wall formation during septation, following UV irradiation, and filaments equal to 50 - 100 normal cells containing apparently normal nuclear bodies, are produced (Howard-Flanders *et al.*,1964 b; Alder & Hardigree, 1965). The rate of DNA, RNA and protein synthesis in growing filaments is normal and the quantities and ratios are the same as in normal cells (Deering, 1958). If a *lon*$^+$ allele is introduced into an irradiated *lon* cell, normal cell division takes place (Walker & Pardee, 1968). In irradiated *lon* cultures, many filaments eventually do divide, others grow to a critical length and then lyse (Kantor & Deering,1966).

Similarities between prophage induction and filament formation were pointed out by Witkin (1967). Both are mass effects caused by exposure to low fluences of radiation or other agents inhibiting DNA synthesis. Fluences that produce 10 - 20 pyrimidine dimers per cell are sufficient to induce filament formation. The production of pyrimidine dimers appears to be the effective lesion as both effects are prevented by photoreactivation. Protein synthesis is required for each to occur and chloramphenicol added after UV irradiation prevents them from occurring. Witkin (1967) hypothesized that UV irradiation brought about the derepression of an operon leading to the formation of a protein which inhibits cell division, and as long as the operon remains derepressed and growth continues, filaments will be formed. When the operon is once more repressed, division of the filament occurs. If, however, the filament reaches a critical length before repression occurs, then recovery is not possible and death results. In addition, both filament formation and prophage induction only occur in *rec* A$^+$ and *lex*$^+$ cells (Green *et al.*, 1969; Donch *et al.*, 1968). The division of filaments, leading to colony formation can be brought about by a number of pre- and post-irradiation treatments which cause a retardation in growth and DNA synthesis, and allow increased repair and recovery to occur (Swenson, 1976). Division of filaments leading to increased survival in irradiated *lon* cells can be enhanced by the addition of cell extracts from *E.coli* B/r, supporting the idea that *lon* cells are defective in a cell division promotion substance (Fisher *et al.*, 1969).

8.1.2 THE USE OF INHIBITORS TO INFLUENCE THE RECOVERY OF UV IRRADIATED CELLS

The use of inhibitors, particularly those which are known to inhibit DNA repair processes, have provided valuable insights into the recovery processes occurring in UV irradiated cells. Both caffeine and acriflavine are known to inhibit repair processes in bacteria (Sauerbier, 1964; Witkin, 1963; Feiner & Hill, 1963). Caffeine is a purine analogue and its action inhibits excision-repair preventing the formation of single-strand breaks, when added immediately after irradiation. If caffeine is added after single-strand breaks have already been formed the excision repair process is no longer inhibited (Fong & Bockrath, 1979). Caffeine also causes an increased delay in cell division and the delay is in proportion to the concentration (Witkin, 1959). Caffeine binds only to irradiated DNA and when bound, interferes with the binding of photoreactivating enzymes to dimers in the DNA (Domon *et al.*, 1970; Harm, 1970). Caffeine does not affect post-replication repair processes (Fong & Bockrath, 1979). As a consequence of its effect on excision repair, caffeine also inhibits liquid holding recovery and host cell reactivation and causes a decrease in cell survival and an increase in the mutation rate in the survivors (Witkin, 1959).

Acriflavine has also been extensively used as an inhibitor of excision repair, however the action of acriflavine appears to be more complex and is not as well understood as caffeine. When acriflavine or other acridine dyes are added to cells before UV irradiation they have a quenching action, and act to protect the DNA by preventing the formation of dimers (Webb & Petrusek, 1966; Setlow & Carrier, 1967; Sutherland & Sutherland, 1969). Setlow (1964) found that the rate of dimer excision was reduced in the presence of acriflavine and Fong & Bockrath (1979) showed that acriflavine inhibited the formation of single-strand breaks in the DNA which occur as a result of the excision repair process. They also found that acriflavine inhibited post replication repair in addition to excision repair, which confirms early reports that excision repair mutants (*uvr*$^-$) also show a decrease in survival in the presence of acriflavine (Alper, 1963; Harm, 1967). Through its effect on DNA repair, acriflavine also inhibits liquid holding recovery and host cell reactivation and causes a decrease in cell survival and a marked increase in the mutation rate (Witkin, 1961; 1963; Alper, 1963; Harm, 1967).

Rossman *et al.*, (1975, 1977) reported that sodium arsenite caused a decrease in the survival of UV irradiated *E.coli* cells and they put forward evidence suggesting that sodium arsenite inhibits a *rec* A dependent step in DNA repair in *E.coli* K-12.

The effects of chloramphenicol on the recovery of irradiated cells has been found to be varied and complex (Swenson, 1976). Pre-irradiation treatment with this antibiotic gave protection against UV radiation in *E.coli* B/r cells and a number of repair deficient mutants (HCR$^-$, *uvr*$^-$, *rec*$^-$) (Rudé and Doudney, 1973). They suggested that this effect was produced by causing a delay in the re-initiation of DNA synthesis after irradiation. When *E.coli B* cells were treated with chlorampherical for short periods after irradiation an increase in survival occurred (Gillies & Alper, 1959; Suzuki & Iwama, 1960; Drakulić *et al.*, 1966). However, when *E.coli* B/r and K-12 wild type and *uvr* mutant cells were treated with chloramphenicol a decrease in survival occurred but *rec*$^-$ mutants showed no effect (Alper & Gillies, 1960; Ganesan & Smith, 1972). They proposed that chloromphenicol interferes with *rec B* function which is dependent on protein synthesis. The increased survival of *E.coli B* is dependent on a functional excision repair system and appears to be related to the increased survival which occurs under suboptimal conditions in this strain (Alper & Gillies, 1958).

A number of other compounds which affect the survival of UV irradiated cells have been investigated, and in most cases the interpretation has been that interference with repair processes was involved. Although the effects that these compounds produce may be no less important than the more extensively studied inhibitors, their effects and modes of action are still poorly understood (See Swenson 1976 for review).

8.1.3 LIQUID HOLDING RECOVERY

When UV irradiated *E.coli* B or B/r cells are held in a non-nutrient buffer for a number of hours before plating onto nutrient medium, they show a much higher level of survival than if plated immediately. Roberts & Aldous (1949) were the first to report this phenomenon known as liquid holding recovery (LHR), and using *E.coli* B they obtained large recovery effects using a variety of buffers which inhibited growth and cell division during the holding period. This recovery effect was found to be almost completely absent in the UV sensitive mutant B_{s-1} (Castellani *et al.*, 1964; Harm, 1968a). The LHR effect has also been shown to occur in yeast cells (Patrick *et al.*, 1964).

Repair competent *E.coli* K-12 and its *uvr*, *rec* B and *rec* C derivatives do not exhibit LHR. However, Ganesan & Smith (1969) showed that LHR did occur in *rec* A strains of *E.coli* K-12. Using different multiple repair defficient strains they showed that the uvr^+ genes were essential for LHR but LHR was only observed in *rec* A cells. The requirement for the uvr^+ genes and the fact that LHR could be inhibited by caffeine strongly suggested that LHR is brought about by excision repair (Ganesan & Smith, 1968a) and was confirmed by Setlow & Carrier (1964) who showed that the excision of dimers occurs during LHR. Recent work done by Tang & Patrick (1977b) has shown that excision repair alone is sufficient to effect LHR in *rec* A strains, and the later steps in the excision repair processes are carried out primarily if not exclusively by DNA polymerase I. Jagger *et al.*, (1964) proposed that LHR caused a growth delay in cells on the plating media after liquid holding, thereby allowing extra time for repair of DNA to occur. However, a series of experiments carried out by Harm (1966) showed that most, if not all of the recovery processes occurred in the liquid holding medium. In these experiments he plated out irradiated cells onto solid culture media with and without caffeine, both before and after liquid holding in buffer. The cells plated onto caffeine before liquid holding showed a ten-fold increase in sensitivity while those plated onto caffeine after liquid holding showed virtually the same survival as those plated onto media without caffeine. By adding caffeine to the holding buffer he was able to inhibit LHR, and when these cells were plated onto caffeine plates they showed a further decrease in survival. He also obtained similar results with acriflavine.

Ganesan & Smith (1968a, 1968b) showed that low concentrations of yeast extract (0,075%) added to liquid holding buffer almost completely inhibited LHR without delay, in *rec* A strains of *E.coli* K-12. A similar observation was made by Alper & Gillies (1960) using *E.coli* B, they showed that cells held in buffer showed higher survival than those held in broth. Ganesan & Smith (1968a) found that extensive degradation of DNA occurred when yeast extract was present. They also found that the inhibitory action of yeast extract on LHR could be completely prevented if chloramphenicol was also added to the holding buffer (Ganesan & Smith, 1968b). Under these conditions the degradation of DNA was less but was still higher than it was in buffer alone. Thus DNA degradation and its prevention does not account entirely for the action of yeast extract and chloramphenicol on LHR in *rec* A cells. Recently Tang *et al.*, (1979) have suggested that LHR depends upon the balance between repair and DNA turnover. They have shown that in non-irradiated *E.coli* B/r there is continuous DNA degradation and resynthesis without a net change in cellular DNA content in cells held in buffer. This constant DNA turnover probably involves most of the genome and reflects random sites of DNA repair due to the *pol* A dependent excision synthesis repair pathway. Under non-growth conditions it appears that at any given time there is a minimum of one repair site per 6,5 x 10^6 daltons DNA, each repair site is at least 160 nucleotides long. They found that the amount of DNA degradation is not influenced by prior exposure to UV radiation but the synthetic activity is decreased with increasing fluences. They suggest that when sites of DNA turnover occur opposite cyclobutyl dimers in UV irradiated cells, repair of the dimers is prevented. This implies that both beneficial and deleterious processes take place in irradiated buffer held cells and that cell survival depends on the delicate balance between DNA turnover and repair of UV damage.

LHR has been shown to overlap with photoreactivation (PR) in *E.coli* (Harm, 1968a; Moss & Davies, 1974). Photoreactivation is able to reverse the lethal effects of UV radiation by cleaving dimers *in situ*. If PR treatment is given before LHR a proportion of the dimers are repaired before LHR occurs and survival remains relatively constant. When PR treatments are given after LHR, there is a rapid decrease in the overlapping effect and less and less of the dimers are repairable by PR (Harm, 1968a). This appears to be due to the production of single-strand breaks adjacent to the dimers caused by the excision repair system, making the lesions irrepairable by PR treatment.

8.1.4 FLUENCE RATE DEPENDENT RECOVERY

In addition to LHR, a second phenomenon involving enhanced survival of irradiated cells in a non-nutreint medium is dependent on the fluence rate. Known as fluence rate dependent recovery (FRR) it was first reported by Harm (1968b). He showed that for a given UV fluence the survival of *E.coli* B or B/r was much greater if they were irradiated at very low fluences for a long period of time, than if the same amount of UV radiation was given at a high fluence for a short time. Later work by Tang & Patrick (1977a) showed that at low fluence rates cells appear to be able to repair the resulting photochemical lesions by the excision repair process almost as fast as they occur.

8.1.5 MINIMAL MEDIUM RECOVERY

Another way of enhancing the recovery of colony forming ability after UV irradiation in some strains of *E.coli* is by plating the cells on minimal medium rather than on a complex medium. This effect was first discovered by Roberts & Aldous (1949) using *E.coli* B. It was also found to occur in repair deficient *uvr* and *rec* mutants of *E.coli* K-12 by Ganesan & Smith (1968b, 1969, 1970), who called the phenomenon minimal medium recovery (MMR). Alper & Gillies (1960) found that the survival of irradiated *E.coli* B was maximal where growth was the slowest and the richer the medium used for plating the cells, the lower was the survival rate. In MMR in *E.coli* B the same type of repair processes appear to be responsible for the recovery that operates in other sub-optimal conditions such as LHR. In *E.coli* B/r and K-12 slower growth does not lead to increased survival and no MMR occurs.

8.1.6 STARVATION OF ESSENTIAL NUTRIENTS

The starvation of auxotrophic strains of *E.coli* of essential nutrients before or after UV irradiation produces different effects in different strains. The differences in the levels of survival that have been observed appear to be due to many complex factors which are not clearly understood (Swenson, 1976).

Gillies (1961) showed that an increase in viable cells occurred when auxotrophic mutants of *E.coli* B were deprived of essential nutrients after irradiation. In *E.coli* K-12 auxotrophic mutants treated in the same way a marked drop in viability occurs (Kos *et al.,* 1965). In *E.coli* B/r a mixed set of responses occurred depending on which nutrients were involved (Forage & Gillies, 1969). The results obtained with *E.coli* B were consistent with other situations, where conditions which inhibit growth and synthesis after UV irradiation brought about an increase in viability.

Most of the studies which have been carried out on the effects of starvation on auxotrophic mutants prior to UV irradiation have used *E.coli* B/r strains. In these strains a marked starvation induced resistance enhancement (SIRE) occurs (Hanawalt, 1966; Billen & Bruns, 1970b; Rudé & Doudney, 1973). Starvation in thymine-requiring strains however, led to a marked decrease in survival (Cohen, 1971; Anderson & Barbour, 1973). Sedliaková *et al.,* (1973) reported that the excision of dimers was not responsible for the increase in survival and excision processes may be retarded or inhibited in starved cells. Mašek *et al.,* (1971) reported that a decrease in the degradation of DNA appeared to be involved and they suggested that pre-starvation inhibits the action of exonucleases and prevents large gaps being formed in the DNA which would be difficult to repair. Work done by Rudé & Doudney (1973) suggested that starvation favours the recovery of viable cells because the reinitiation of DNA synthesis occurred more readily under these conditions, and that dimers were repaired by post-replication repair (Sedliaková *et al.*, 1973).

8.1.7 THE EFFECT OF PLASMIDS ON RECOVERY OF CELLS AFTER UV IRRADIATION

The presence of plasmids in irradiated cells may cause increased resistance to UV irradiation, increased sensitivity, or have no effect (Howarth, 1965; Siccardi, 1969; MacPhee, 1972; Lehrbach *et al.*, 1978). In *E.coli*, *S. typhimurium*, and *Ps.aeruginosa* the product of the *rec* A gene is required in cases of both increased and decreased survival, and is independent of *uvr* and *pol* A gene products. In some plasmids the *rec* A dependent pathway has been found to be sensitive to sodium arsenite and in others to be insensitive (Lehrbach *et al.*, 1978). The requirement for the *rec* A gene product in R plasmids affecting UV sensitivity suggests that some UV inducible process may be involved (Swenson, 1976).

8.1.8 THE EFFECTS OF PRE-IRRADIATION TREATMENTS ON RECOVERY OF UV IRRADIATED CELLS

The effects of a number of pre-irradiation treatments, including the effect of inhibitors and starvation of essential nutrients, on the recovery of irradiated cells has already been mentioned. In those bacteria so far studied, actively growing cells appear to be more sensitive to UV irradiation than stationary phase cells (Swenson, 1976; Webb, 1977). Morton & Haynes (1969) found that in early exponential phase, cells were more resistant than in late exponential phase. They showed that sensitivity increased to a maximum during the late exponential phase and then became less sensitive when the cells entered stationary phase. Ginsberg &

Jagger (1965) showed that the amount of damage induced by UV radiation was constant and independent of the growth phase, the difference in sensitivity during the different growth phases appear to be due entirely to differences in the efficiency of repair. The fact that changes in sensitivity are due mainly to changes in the size of the shoulder, rather than changes in the final slope of the curve, support this view. Morton & Haynes (1969) suggested that variations in survival during the growth cycle are caused by differences in the intracellular concentrations or activities of repair enzymes. Tyrrell *et al.* (1972) found that the level of activity of *rec* system enzymes remained fairly stable during the exponential phase but levels of activity of the excision repair enzymes decreased towards later exponential and then began to increase again in stationary phase. Attempts to study the UV sensitivity of individual *E.coli* cells during the various stages in the cell cycle have given conflicting results. Helmstetter & Uretz (1963) reported a sharp drop in sensitivity in the middle and at the end of the division cycle and Kubitschek *et al.* (1973) found that no difference in sensitivity occurred during the cell cycle.

When *E.coli* cells are exposed to near UV radiation between 300 - 400 nm prior to far UV radiation, they are protected against the lethal effects of the latter (Weatherwax, 1956; Jagger, 1960; Jagger *et al.*, 1964). The photoprotection effect of near UV light is brought about by the inhibition of growth and division in cells treated with near UV light (Jagger *et al.*, 1964; Phillips *et al.*, 1967). Work by Ramabhadran *et al.* (1975) suggested that 4-thiouridine in transfer RNA was the chromophore and target for growth delay caused by near UV radiation. The growth delay in cells after near UV irradiation allows cells a longer time for the repair of far UV damage to occur (Jagger *et al.*, 1964). The recovery mechanism appears to be similar to that operating in LHR and the delay allows time for the single strand breaks produced during excision repair to be closed before DNA replication and growth are resumed (Swenson *et al.*, 1975). There is no difference in the amount of damage caused by far UV irradiation in unprotected and photoprotected cells, and both contain the same number of photoproducts in their DNA after far UV irradiation (Swenson *et al.*, 1975).

8.2 MATERIALS AND METHODS

8.2.1 MEASUREMENT OF CELL SURVIVAL

The growth and irradiation procedures have been described in 7.2.2 and 7.2.3. The survival curves of cell suspensions irradiated with increasing fluences of UV radiation were determined using the methods described in 7.2.4. Liquid holding treatments of 2,5 h at 30°C were used to ensure maximum recovery, and survival curves were determined, both before and after each specific type of liquid holding treatment. Time course viability curves were obtained using cell suspensions irradiated with a fluence of 30 J m^{-2} which gave *c.* 1% survival when cells were plated onto BHI agar after aerobic irradiation.

Irradiated cells (1 ml) were transferred to liquid holding media (9 ml) and held for a 12 h period at 30°C. Viability was determined by removing 0,1 ml samples at fixed time intervals (usually

0,1,2,3,6 & 12 h), diluting in Ringer solution and plating 0,1 ml of the appropriate dilutions onto BHI plates. Each sample was plated onto a minimum of 4 agar plates, and the number of surviving colonies were counted after incubation for 48 h at 37°C under anaerobic conditions. The mean of the results were used to calculate the surviving fraction in each sample. In the experiments involving irradiation and holding under anaerobic conditions, all procedures were carried out in an anaerobic glove box.

8.2.2 POST IRRADIATION TREATMENT

A variety of liquid holding media were used in investigating the effects of different post-irradiation treatments on the recovery of irradiated *B. fragilis* cells. The non-nutrient solutions used were selected for their ability to hold cells under non-growth conditions with the minimum loss in viability. The holding properties of a number of solutions, under both anaerobic and aerobic conditions were determined (See Chapter 2.3.1). For liquid holding under anaerobic conditions,both the anaerobe solution (AS) and the minimal salts solution (MS) proved satisfactory and for liquid holding under aerobic conditions, Ringer solution (RS) was used. In order to investigate the effect of nutrientson LHR, RS containing yeast extract (0,75%) and peptone (1,5%) and BHI holding media were used. The m.i.c. of the inhibitors used in the investigation of cell recovery, were determined for both liquid and solid media as variations were found to occur under the two conditions. Inhibitors were either incorporated into the liquid holding solutions or into the plating media at concentrations slightly less than the m.i.c. In addition to cell suspensions prepared in the usual way (7.2.1), in some experiments cells were washed and pre-held in RS for 2 h before irradiation. In all experiments excepting those involving minimal medium recovery, cells were plated onto BHI medium or BHI medium containing inhibitors, to determine the number of survivors.

8.2.3 FILAMENT FORMATION

In order to investigate filament formation in irradiated *B. fragilis* cells, cell suspensions were prepared as described in (7.2.1), and then irradiated under both anaerobic and aerobic conditions with UV fluences of 8, 16 & 32 J m^{-2}. Unirradiated cell suspensions were used as controls. Cell suspensions were then either transferred to BHI broth immediately, or held for 2,5 h at 30°C in buffer under anaerobic or aerobic conditions, and then transferred to BHI broth. The OD of the cultures were measured over a 24 h period and samples of cells were examined at fixed time intervals, for filament formation, using phase contrast optics. The proportion of filaments present in the population was determined using a slide counting chamber with THOMA type ruling (Gallencamp).

8.3 RESULTS

8.3.1 GROWTH AND FILAMENT FORMATION AFTER UV IRRADIATION

Observations suggest that *B. fragilis* (PS 1) cells showed some delay in growth after UV irradiation. Colony formation on solid medium was delayed after UV irradiation and a variation in the size of the colonies occurred after exposure to high fluences of UV radiation. In irradiated cells inoculated into liquid medium, an apparent delay in the resumption of growth due to the reduction in the number of viable cells in the inoculum occurred, and must be distinguished from a delay in growth caused by the inhibition of cell division and DNA replication in viable cells. In cells subjected to an UV fluence of 32 J m^{-2}(*c*.1% survival), a growth delay of over 7 h occurred (Fig. 8.1). In addition, the actual growth rate appeared to be slightly slower, and the final OD of the culture was lower than in cultures inoculated with unirradiated cells at the same concentration (Fig. 8.1). At lower fluences (8 J m^{-2}) the time taken for the population to reach stationary phase was much more rapid and was only slightly slower than the time taken by unirradiated cells.

In BHI broth, *B. fragilis* (PS1) cells in exponential phase cultures occurred as short uniform rods, which were often in pairs and a very small proportion of short chains and long rods were present. In *B. fragilis* cells inoculated into BHI broth after UV irradiation a very much higher proportion of the cells developed into elongated rods. These cells continued growing so that at the end of the exponential growth phase, long filaments were produced. These filaments were between 10 and 60 times the length of the normal rod-shaped cells. They differed from the filaments produced by *E.coli*, in that there were always a number of septa present in each filament. With increasing fluences of UV radiation there was an increase in the proportion of filaments produced in the population and more filaments appeared to be produced after irradiation under aerobic conditions, than after irradiation under anaerobic conditions (Table 8.1). When cells were held in buffer for 2,5 h there was a decrease in the number of filaments formed and fewer filaments appeared to be produced when cells were held under anaerobic conditions (Table 8.1). An increase in the number of filaments also occurred in the unirradiated

TABLE 8.1 Filament formation in *B. fragilis* cells after UV irradiation and liquid holding under different conditions

Irradiation	Fluence $J\ m^{-2}$	Liquid Holding Treatment	% Filament formation at end of exponential phase
Unirradiated	0	-	0 - 10%
	0	2,5 h anaerobic	15 - 30%
	0	2,5 h aerobic	15 - 30%
Anaerobic Irradiation	8	-	10 - 25%
	16	-	10 - 25%
Aerobic Irradiation	8	-	10 - 25%
	16	-	10 - 25%
	32	-	40 - 55%
	32	2,5 h anaerobic	25 - 35%
	32	2,5 h aerobic	30 - 45%

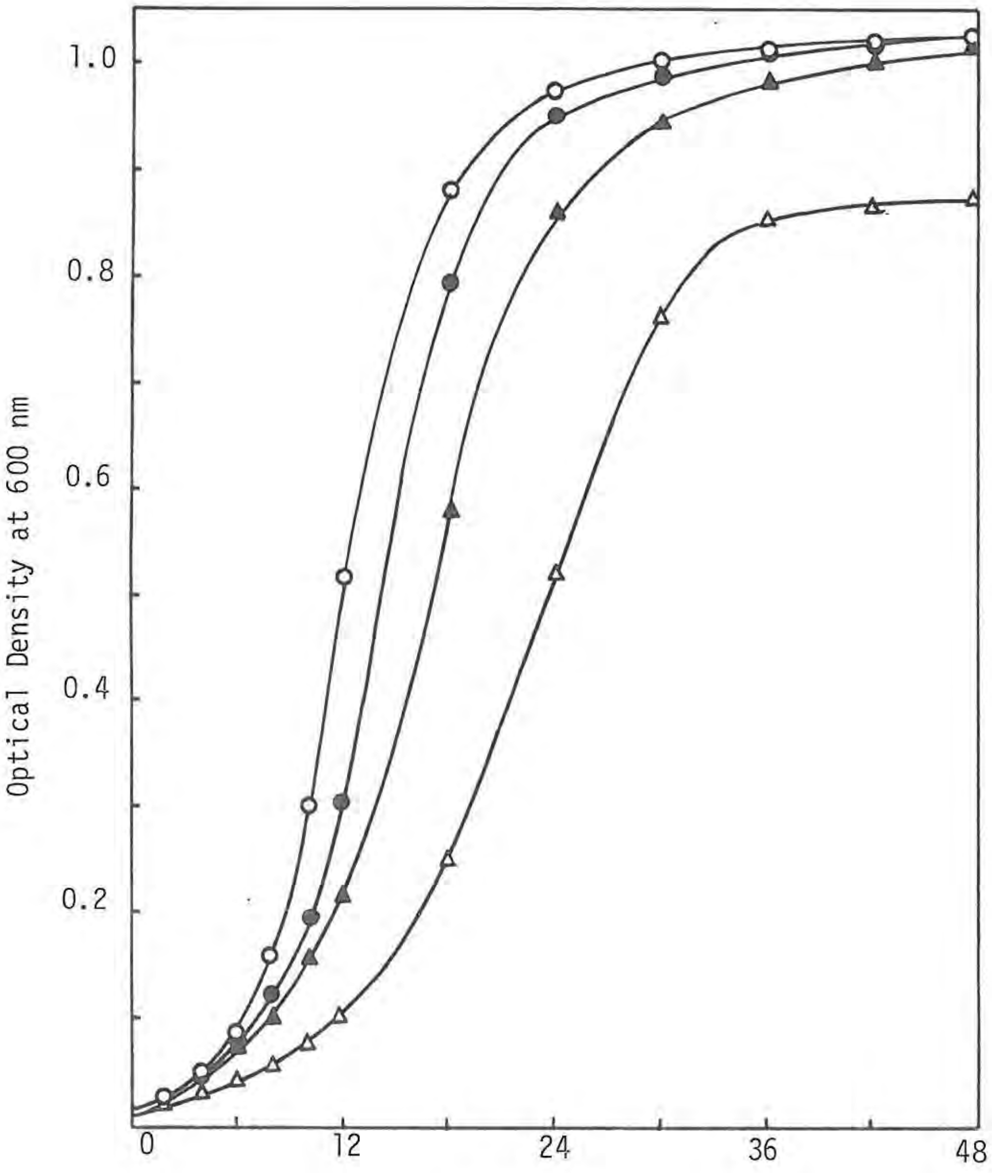


FIG. 8.1: Growth of *B. fragilis* in BHI broth in unirradiated cells (o) and cells irradiated with a fluence of 8 J m^{-2} under anaerobic conditions (●) and under aerobic conditions (▲), and cells irradiated with a fluence of 32 J m^{-2} under aerobic conditions (Δ).

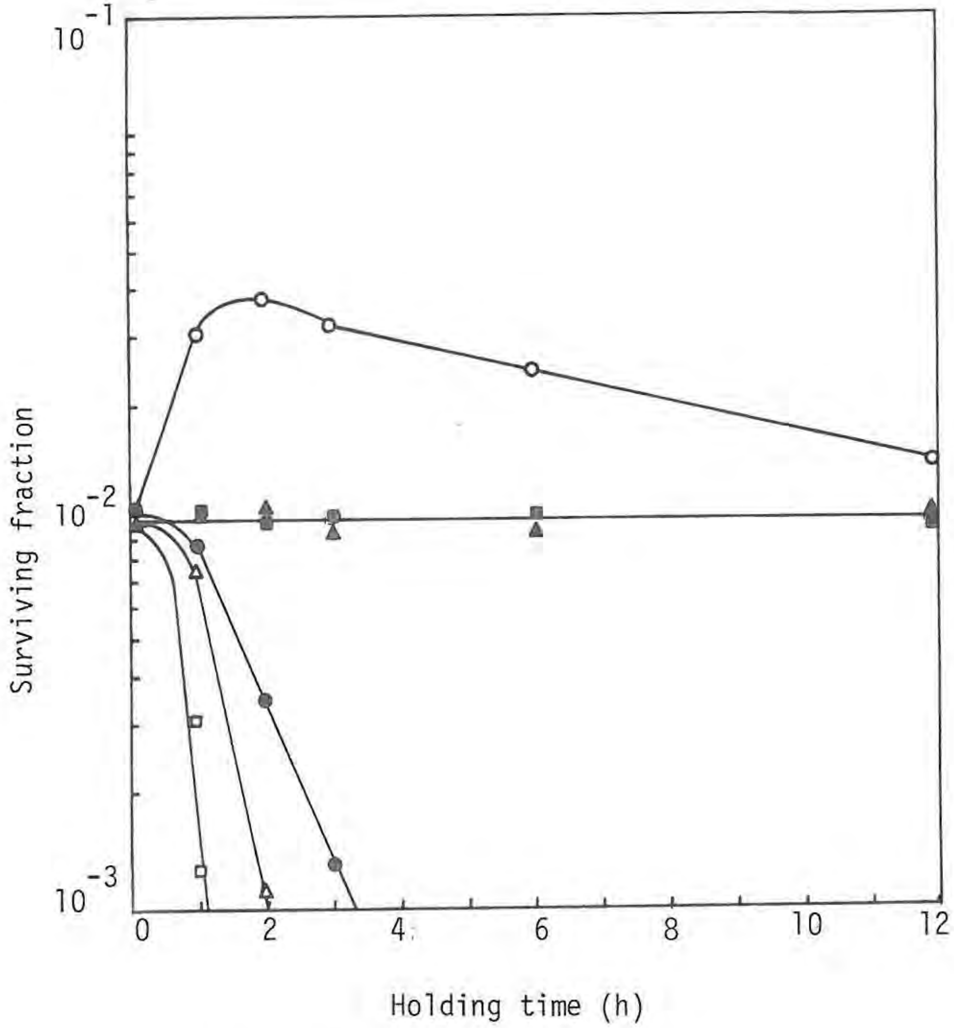


FIG. 8.2: Survival of *B. fragilis* cells irradiated with 30 J m^{-2} (1% survival) under aerobic conditions showing the time course viability curve for cells held aerobically in RS (o), AS (Δ) & MS (□) and cells held anaerobically in RS (●), AS (▲) & MS (■).

control cells held in buffer under both anaerobic and aerobic conditions prior to being transferred to BHI broth (Table 8.1). The significance of filament formation is not clear as the proportion of filaments formed in cell suspensions which were subjected to similar treatments appeared to be variable.

8.3.2 THE RECOVERY OF CELLS AFTER LIQUID HOLDING UNDER ANAEROBIC AND AEROBIC CONDITIONS

Initial experiments indicated that some LHR did occur in *B.fragilis* after UV irradiation. This phenomenon was investigated further and both surivial curves and time course viability curves were determined for irradiated cells held under a variety of conditions. To obtain suitable holding media for these experiments, the holding properties of a number of solutions were tested (Chapter 2.3.2). The anaerobe solution (AS) of Holdemen & Moore (1972) and the minimal salts solution (MS) of Varel & Bryant (1974) were used for holding cells under anaerobic conditions, but had very poor holding properties under aerobic conditions. RS was used for holding under aerobic conditions. No solution was found which could hold cells under both anaerobic and aerobic conditions. LHR was found to occur only under aerobic conditions in *B.fragilis* (PS1) (Figs. 8.2; 8.3; 8.4; 8.5 & 8.6). The time course viability curves for stationary phase *B.fragilis* cells irradiated with a fluence of 30 J m^{-2} under aerobic conditions and then held in RS, AS and MS under both anaerobic and aerobic conditions are shown in Fig. 8.2. When irradiated cells were held in AS or MS under anaerobic conditions, no increase in viability occurred over a 12 h period and the number of surviving cells either remained constant or showed a small drop in viability. Cells held in RS under anaerobic conditions showed a very rapid drop in viability. When irradiated stationary phase cells were held under aerobic conditions in RS a 2 to 7 fold increase in viability occurred. Viability increased rapidly during the first hour of liquid holding and reached a maximum after 2 to 3 h, after which there was a gradual fall in viability during the rest of the 12 h holding period. Cells held in AS or MS under aerobic conditions showed a rapid drop in viability, in some experiments there was a small increase in viability during the first hour of liquid holding followed by a very rapid loss of viability.

The survival curves of stationary phase cells irradiated under both aerobic and anaerobic conditions with increasing fluences of UV radiation and then held for 2,5 h at 30°C in AS under anaerobic conditions are shown in Fig. 8.3. In both cases the cells held under anaerobic conditions showed a very small decrease in survival. When stationary phase cells irradiated under the same conditions were held under aerobic conditions in RS for 2,5 h at 30°C an increase in cell survival occurred, and a DRF ranging from 0,8 to 0,9 at 1% survival was obtained (Fig. 8.4). This increase in survival was due to an increase in the size of the shoulder region of the curve, rather than to a change in the final slope of the curve. When stationary phase cells were pre-held in RS under aerobic conditions for 2 h prior to UV irradiation, followed by a 2,5 h post-UV holding under aerobic conditions, the amount of recovery was increased, and a DRF of 0,78 or less occurred at 1% survival. In exponential phase cells held in RS under aerobic conditions for 2,5 h after UV irradiation under aerobic conditions, the level of recovery was found to be greater than that occurring in stationary phase cells

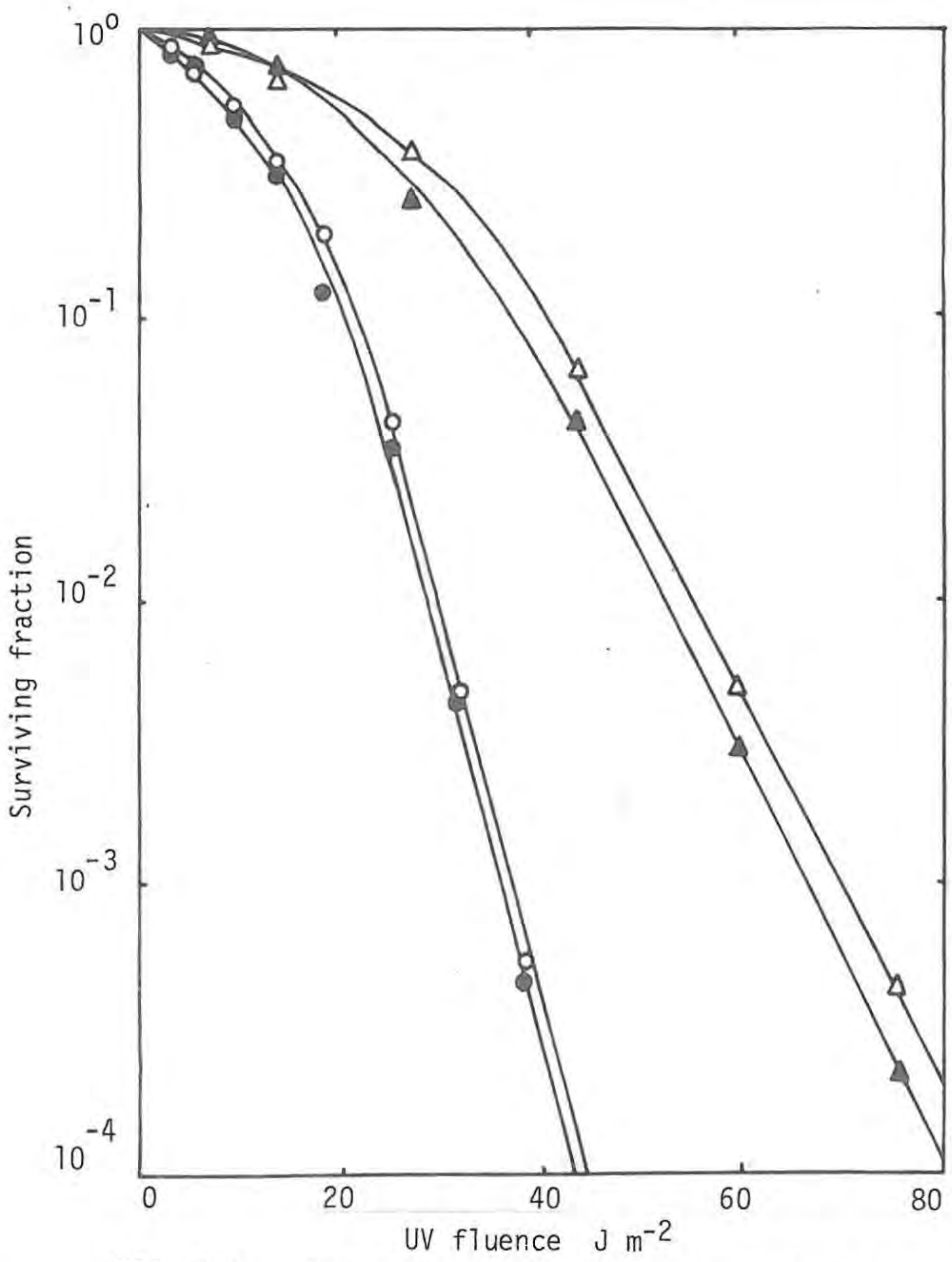


FIG. 8.3: Survival of *B.fragilis* cells after LH under anaerobic conditions. Survival curves of stationary phase cells irradiated anaerobically, plated immediately (Δ) & held for 2,5 h in AS (▲). Survival curves of stationary phase cells irradiated aerobically, plated immediately (o) and held for 2,5 h in AS(●).

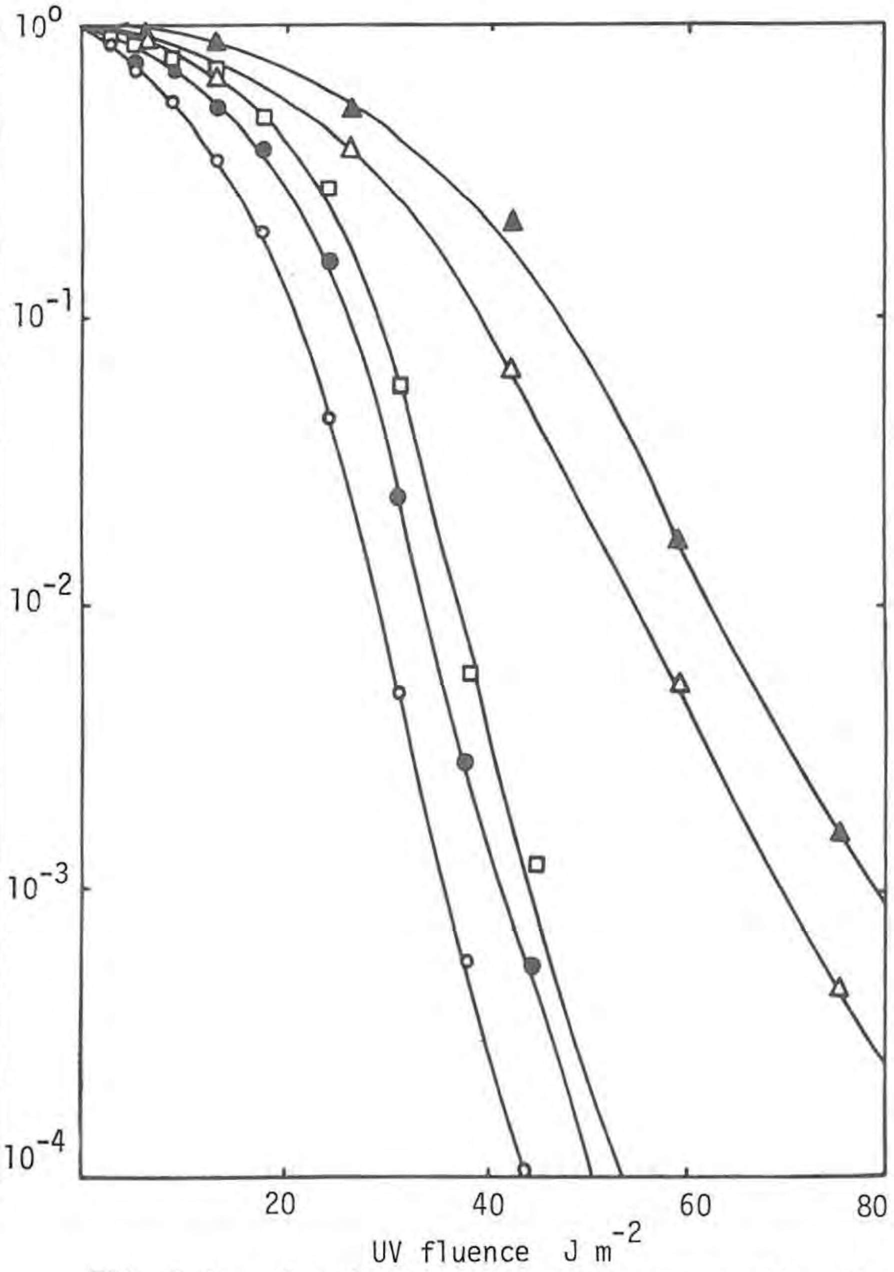


FIG. 8.4: Survival of *B.fragilis* cells after LH under aerobic conditions. Survival curves of stationary phase cells irradiated anaerobically, plated immediately (Δ) held for 2,5 h in aerobic RS (▲). Survival curves of stationary phase cells irradiated aerobically, plated immediately (o), held for 2,5 h in aerobic RS (●), pre-held for 2 h in aerobic RB prior to irradiation and a further 2 h holding in aerobic RS (□).

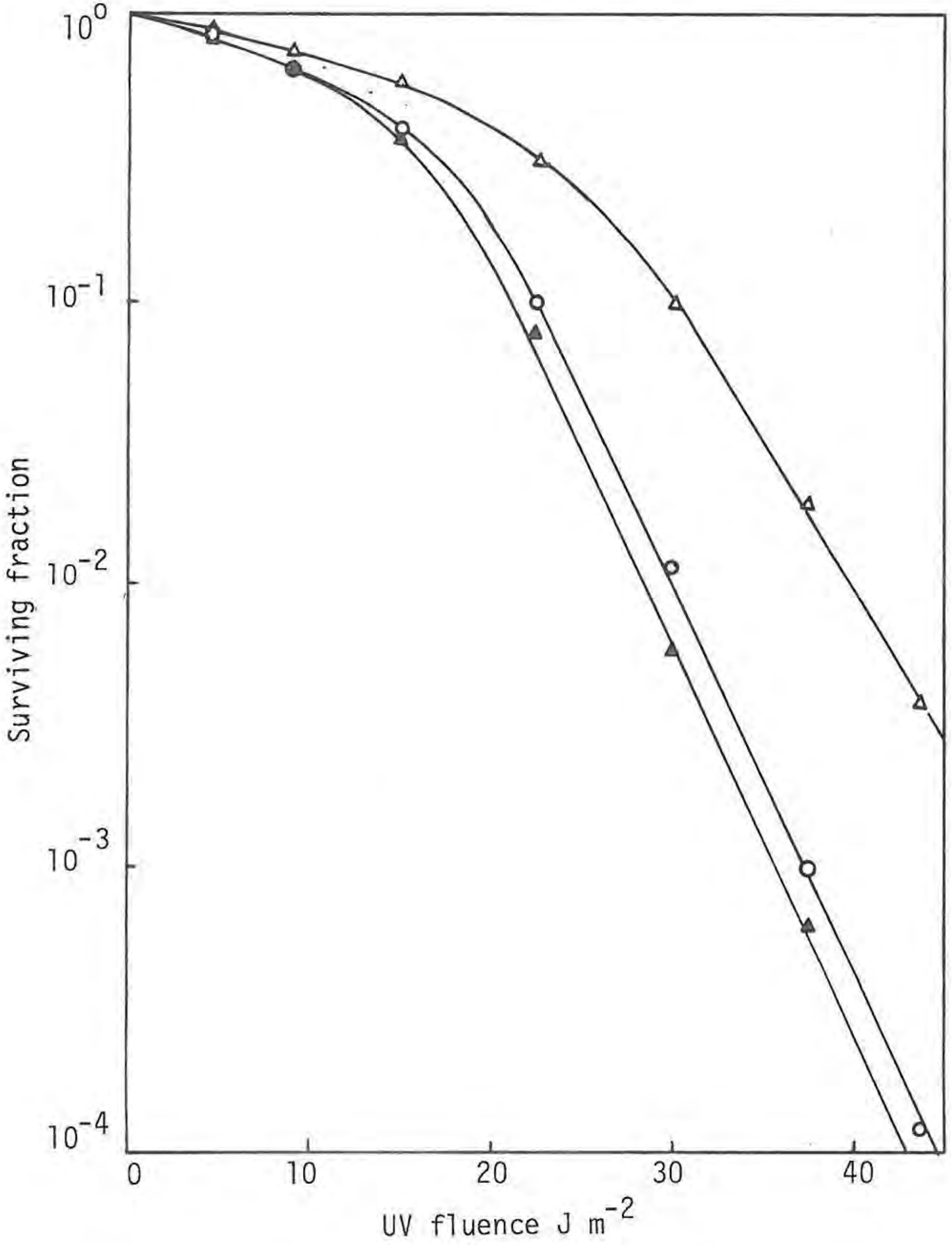


FIG. 8.5: Survival of exponential phase *B.fragilis* cells after LH. Survival curves of aerobically irradiated cells, plated immediately (o), held for 2,5 h in aerobic RS (Δ), held for 2,5 h in AS (▲).

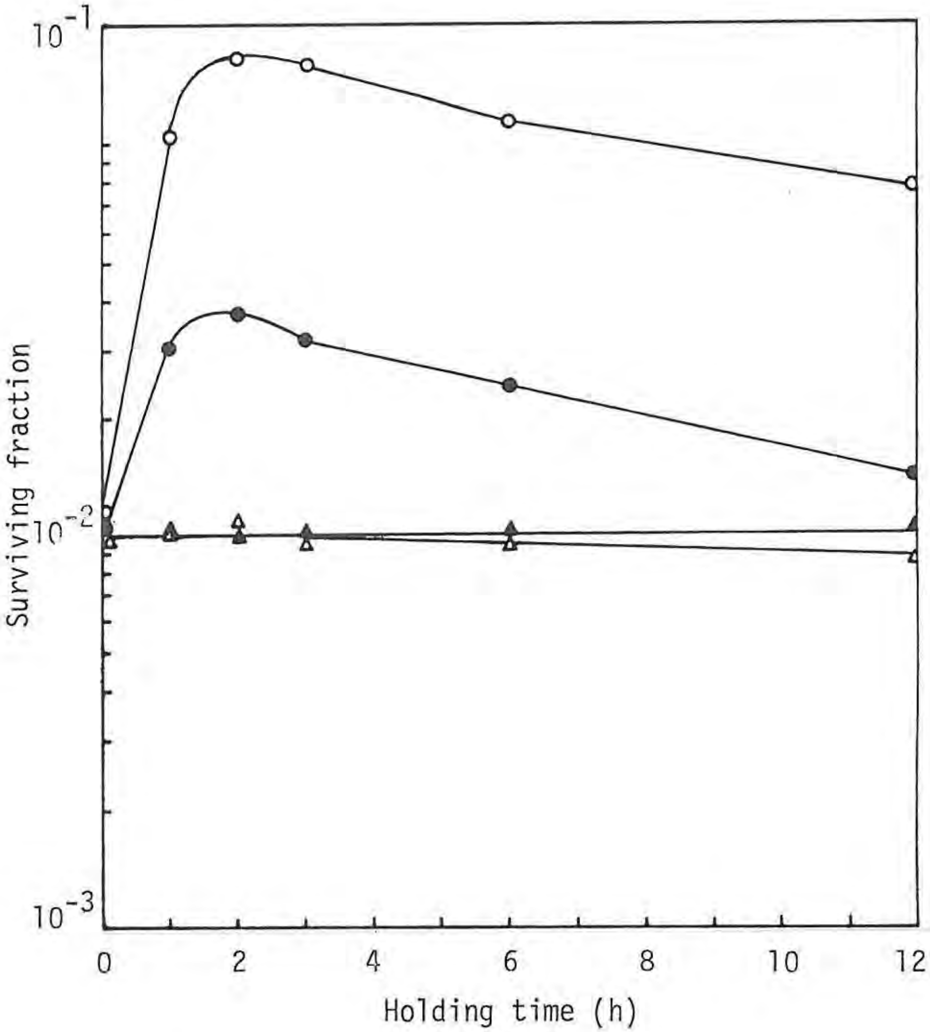


FIG. 8.6: Survival of *B.fragilis* cells irradiated with 30 J m^{-2} (1% survival) under aerobic conditions, showing the time course viability curves for exponential phase (o) and stationary phase (●) cells held aerobically in RS, and exponential phase (Δ) and stationary phase (▲) cells held in AS.

and a DRF of between 0,69 and 0,75 occurred at 1% survival (Fig. 8.5). This increase in survival was reflected by an increase in the shoulder of the survival curve. When irradiated exponential cells were held in AS for 2,5 h under anaerobic conditions, no LHR occurred and there was a slight drop in cell survival (Fig. 8.5). The increased level of recovery in exponential phase cells held in RB under aerobic conditions is also shown in Fig. 8.6. In both stationary and exponential phase cells LHR reached its maximum between 2 to 3 h after irradiation, and then the viability of the cells held in RS showed a gradual decrease. This drop in viability appeared to be directly related to the holding properties of the buffer. When exponential phase cells and stationary phase cells were held in AS after aerobic irradiation no LHR occurred. However, the recovery process was found to be extremely sensitive to the presence of oxygen and if trace amounts were present in the anaerobic holding media a small gradual increase in the number of viable cells occurred over the 12 h holding period.

8.3.3 THE EFFECT OF TEMPERATURE ON LHR UNDER AEROBIC CONDITIONS

Both the rate of recovery and the amount of recovery occurring in irradiated cells held under aerobic conditions was found to be dependent on temperature. The viability of stationary phase cells irradiated with a fluence of 30 J m^{-2} under aerobic conditions and then held in RS under aerobic conditions at different temperatures over a 12 h period is shown in Fig. 8.7. The maximum amount and rate of LHR occurred when cells were held at 37^{o}C. The percentage cell recovery decreased at temperatures above and below 37^{o}C (Fig. 8.8). The rate at which LHR occurred increased as the holding temperature increased. At 4^{o}C the maximum amount of recovery only

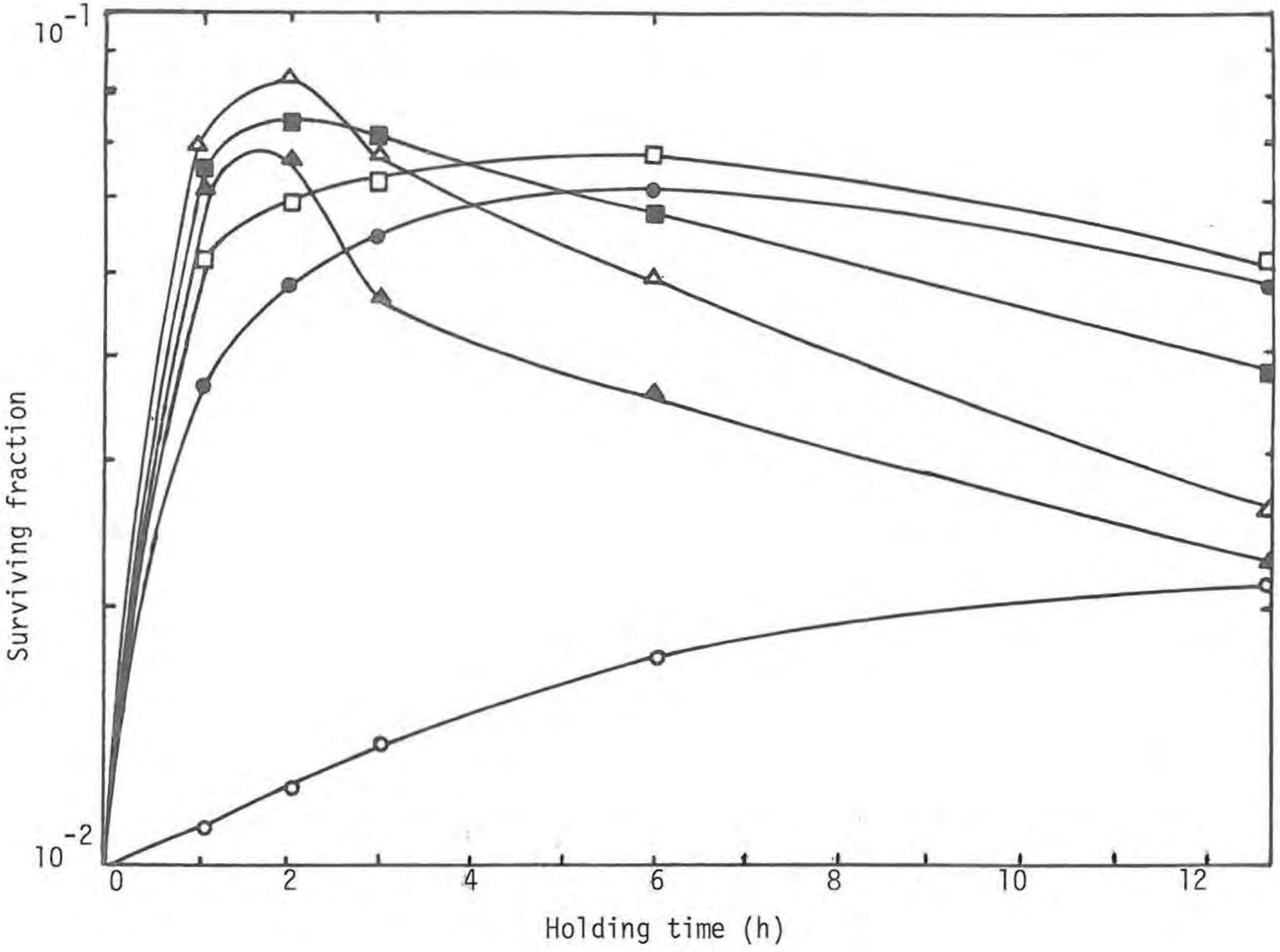


FIG. 8.7: The time course viability curves for stationary phase *B.fragilis* cells irradiated with 30 J m^{-2} under aerobic conditions and held aerobically at 4°C (o), 20°C (●), 25°C (□), 30°C (■), 37°C (△), 42°C (▲).

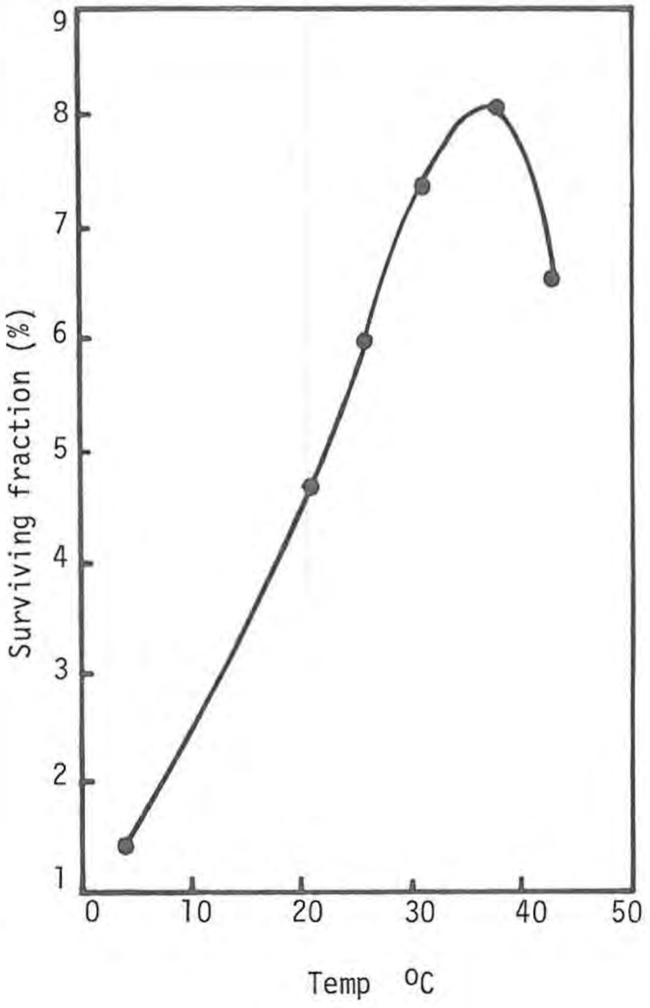


FIG. 8.8: The effect of temperature on LHR showing the % survival after 2 h of cells held at the various temperatures shown in Fig. 8.7.

occurred after 12 h holding. Cells held at 20 and 25^{o}C showed a maximum recovery after *c.* 6 h and at higher temperatures maximum recovery occurred at *c.*2 h. At 4^{o}C the amount of recovery was markedly decreased but the holding property of the buffer appeared to be improved.

8.3.4 THE EFFECT OF NUTRIENTS ON THE RECOVERY OF CELLS HELD UNDER AEROBIC CONDITIONS.

In order to test the effects of nutrients on the aerobic LHR process, peptone (1,5%) and yeast extract (0,75%) were added to RS. In addition the recovery of irradiated cells was also determined in BHI holding medium. None of these holding media supported the growth of unirradiated cells under aerobic conditions, however, LHR was found to occur in all 3 media under aerobic conditions. In irradiated stationary phase cells held in nutrient and non-nutrient holding media, very little difference in viability occurred, and LHR appeared to be only very slightly inhibited in the presence of nutrients (Figs. 8.9 & 8.10). The addition of peptone to RS did however appear to improve its holding properties. In irradiated exponential phase cells a more marked nutrient effect was observed and in both the BHI holding media (Fig. 8.10) and in RS with 0,75% yeast extract (Fig. 8.9) a decrease of between 30 - 40% occurred in the number of viable cells, in the presence of nutrients.

8.3.5 MINIMUM MEDIUM RECOVERY

In addition to LHR, minimum medium recovery (MMR) was also found to occur in *B. fragilis*. In MMR the colony forming ability of irradiated cells is enhanced when they are plated onto minimal

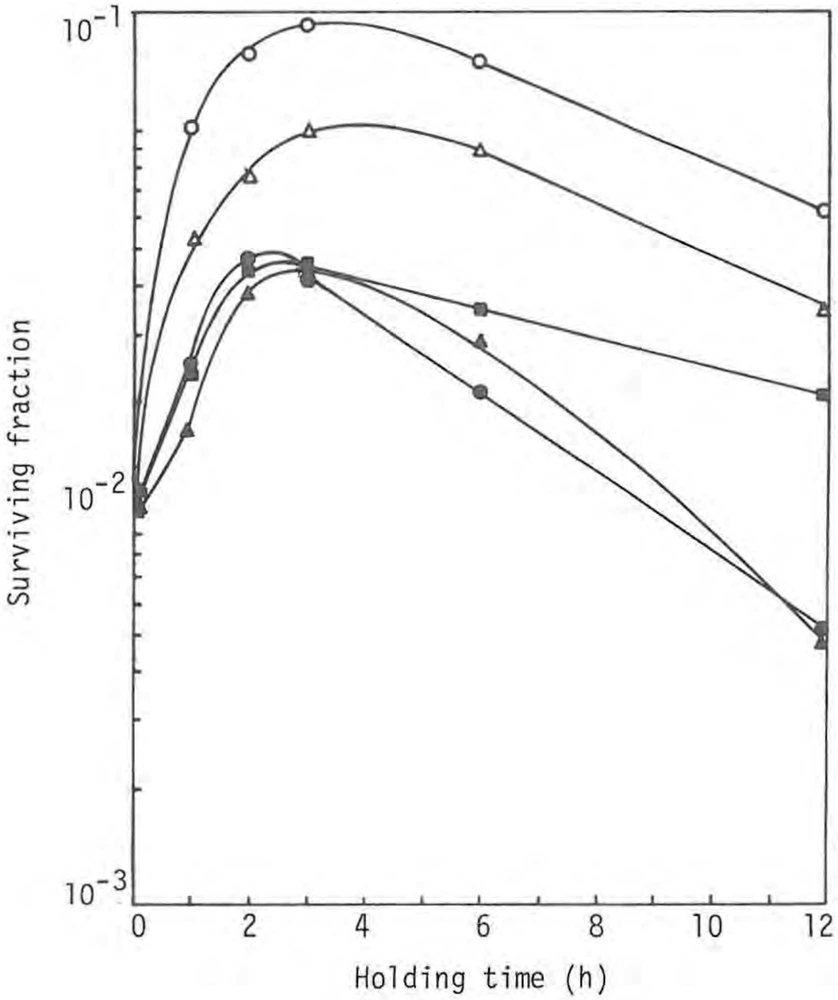


FIG. 8.9: Survival of *B. fragilis* cells irradiated with 30 J m^{-2} (1% survival) under aerobic conditions for exponential phase cells held aerobically in RS (o) and RS+yeast extract (Δ), and for stationary phase cells held aerobically in RS (●) and RS+yeast extract (▲) and RS+peptone (■).

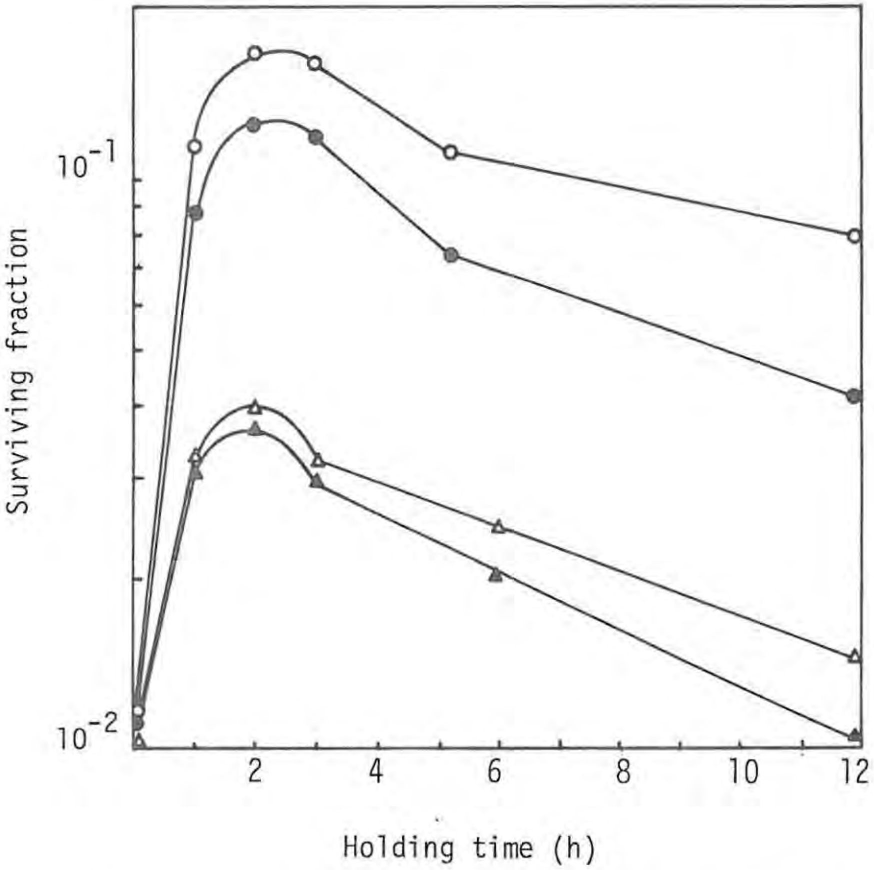


FIG. 8.10: Survival of *B. fragilis* cells irradiated with 30 J m^{-2} (1% survival) under aerobic conditions showing the time course survival curves for exponential phase cells held aerobically in RS (non-nutrient) (o) and BHI buffer (nutrient) (●) and for stationary phase cells held aerobically in RS (Δ) and BHI buffer (▲).

medium instead of a complex medium. The survival curves obtained for exponential phase cells irradiated under aerobic conditions and plated onto both complex medium (BHI agar) and minimal medium, are shown in Fig. 8.11. Cells plated onto minimal medium showed an increase in survival,and a DRF of *c.* 0,75 occurred at 1% survival. The increase in survival obtained after MMR was also reflected as an increase in the shoulder region of the survival curve, and was of the same order of recovery as that obtained by LHR. In addition to plating cells onto minimal medium immediately after irradiation, cells were also plated onto minimal medium after being subjected to liquid holding for 2,5 h in RS under aerobic conditions. Irradiated cells which were given liquid holding treatment and then plated onto minimal medium showed a further increase in survival resulting in a DRF of 0,57 at 1% survival as compared with a DRF of *c.* 0,75 when plated onto minimal medium without a liquid holding treatment. The effect of the two types of post-irradiation treatment were therefore cumulative, and was reflected as a very marked extension of the shoulder region of the survival curve.

8.3.6 THE EFFECT OF CAFFEINE AND ACRIFLAVINE ON THE RECOVERY OF CELLS HELD UNDER AEROBIC AND ANAEROBIC CONDITIONS.

Both caffeine and acriflavine have been shown to inhibit excision repair processes, and LHR in *E.coli*. These two inhibitors were also found to inhibit LHR in *B. fragilis*. The m.i.c. for caffeine and acriflavine were determined both in liquid and in solid media and concentrations slightly lower than the m.i.c. were incorporated

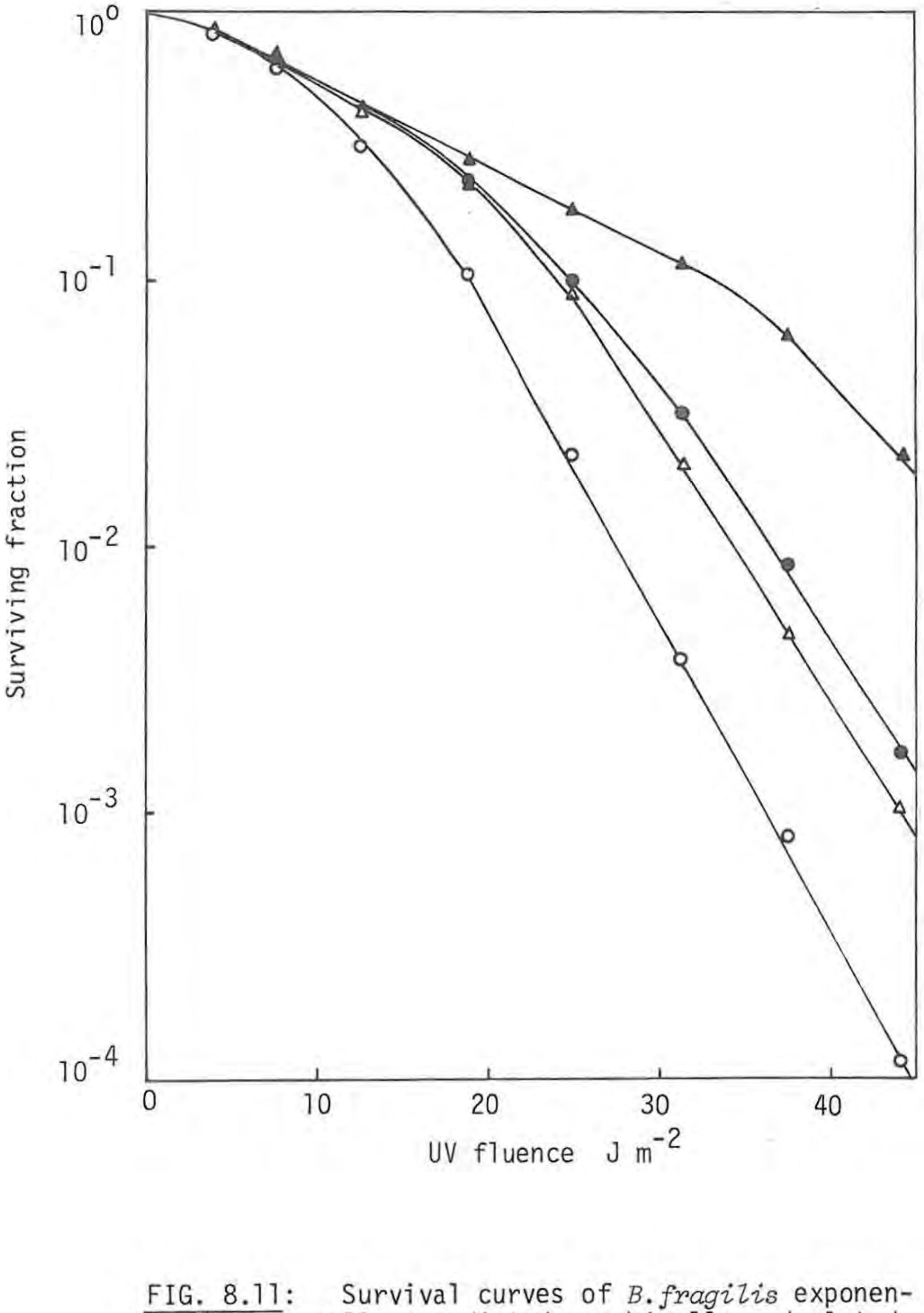


FIG. 8.11: Survival curves of *B.fragilis* exponential phase cells irradiated aerobically and plated onto BHI immediately (o) and after 2,5 h LH in RS (●), and plated onto MM immediately (Δ) and after 2,5 h aerobic LH in RS (▲).

into the RS and AS holding buffers (Acriflavine 0,25; 0,5; 2,5 μg ml^{-1}, caffeine 2,5; 5 mg ml^{-1})and plated onto the plating media (caffeine 1 mg ml^{-1}). When acriflavine was incorporated into liquid holding buffers at concentrations > 10-fold below the m.i.c. (0,25 μg ml^{-1}), not only did it inhibit LHR under aerobic conditions but it also brought about a rapid drop in cell viability (Fig. 8.12). Acriflavine was not used in later experiments because of the rapid drop in viability which occurred in the presence of this inhibitor under aerobic conditions. The addition of caffeine to liquid holding buffer was found to inhibit LHR under aerobic conditions (Figs. 8.12 and 8.13). In the experiment to determine the time course viability curves in the presence of caffeine a small amount of recovery occurred at low concentrations (2,5 mg ml^{-1}) but at higher concentrations a loss in viability occurred (Fig. 8.12). Similar results were obtained in the survival curve experiments, where cells held in the presence of caffeine under aerobic conditions showed almost complete inhibition of LHR, and cells held under anaerobic conditions showed a DEF of *c.* 1,5 at 1% survival (Fig. 8.13). The differences in the level of survival obtained under the various conditions were due to changes in the shoulder of the survival curves.

In an attempt to determine whether the repair processes responsible for LHR in *B. fragilis* occurred entirely in the liquid holding buffer, as they do in *E.coli*, cells were first held in buffer for 2,5 h after irradiation before plating onto BHI agar containing caffeine. The results were compared with those obtained for cells plated onto BHI after liquid holding and

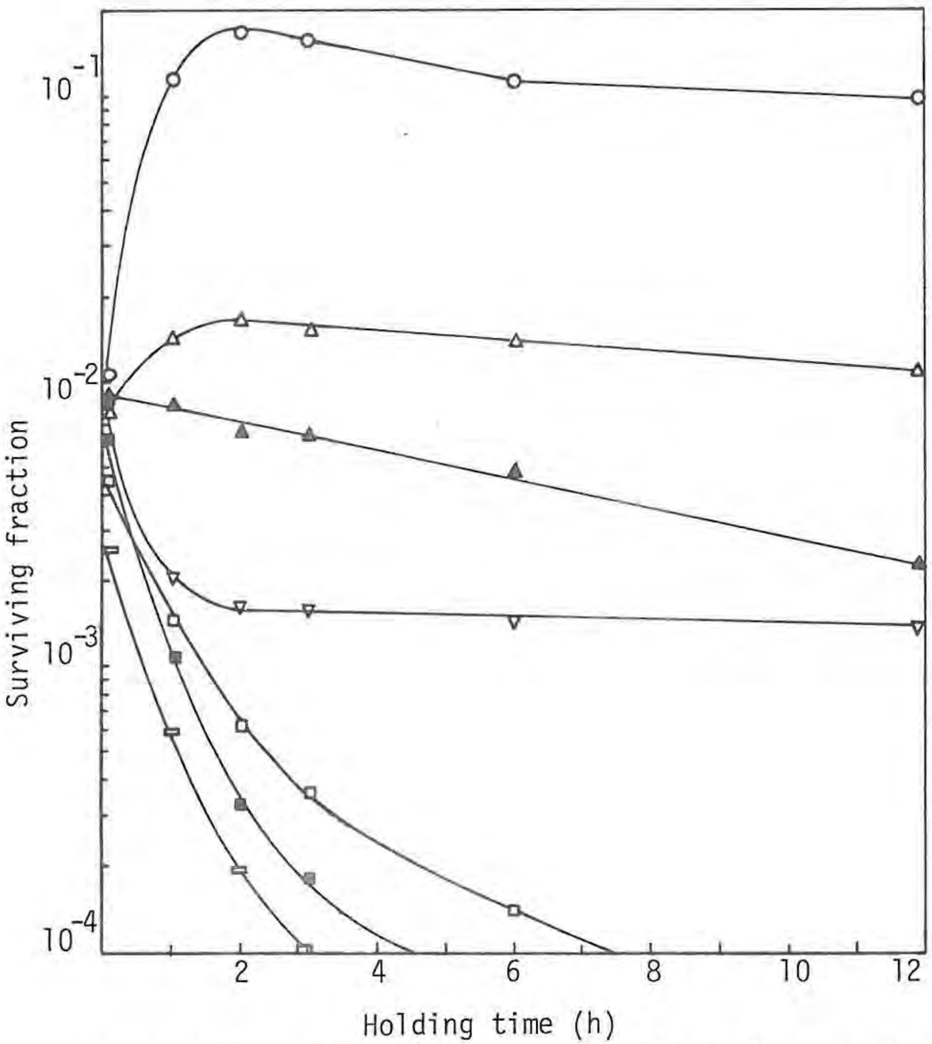


FIG. 8.12: Effect of caffeine and acriflavine on LHR in *B.fragilis* exponential phase cells irradiated aerobically with 30 J m^{-2} and held aerobically for 0 - 12 h in RS (o) and RS containing 0,25 (Δ), 0,5 (▲) and 5,0 mg m^{-1} caffeine (∇), and 0,5 (□), 1,0 (■), and 2,5 µg ml^{-1} acriflavine (▭).

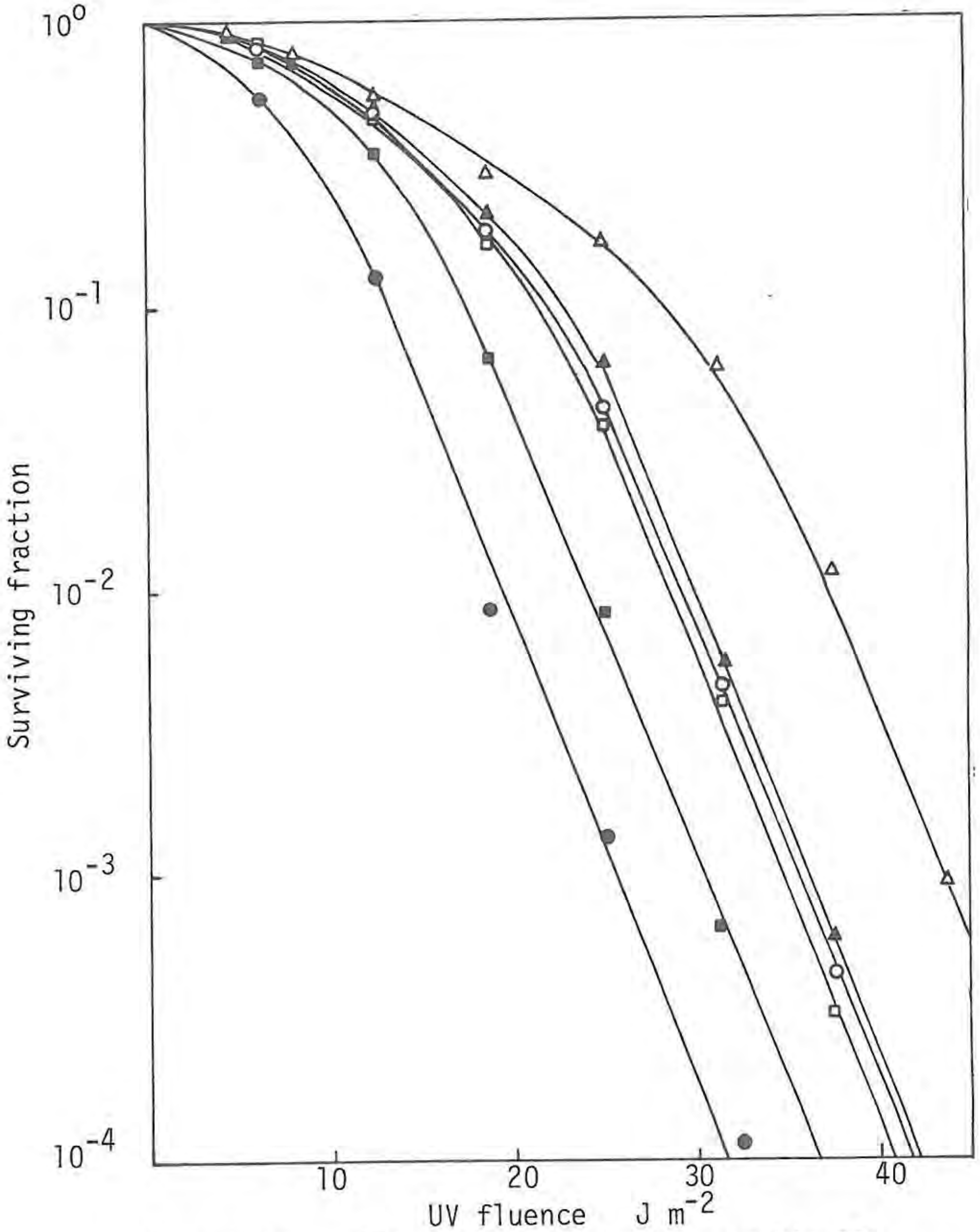


FIG. 8.13: Effect of caffeine on aerobically irradiated exponential phase *B.fragilis* cells held under aerobic and anaerobic conditions for 2,5 h. plated onto BHI at 0 h.(o), and onto caffeine at 0 h (●), held aerobically in RS for 2,5 h (Δ), and aerobically in RS+caffeine for 2,5 h (▲), and held anaerobically in AS for 2,5 h (□), and anaerobically in AS+caffeine for 2,5 h (■).

cells plated directly onto caffeine plates after irradiation. The survival level of cells plated onto caffeine immediately after UV irradiation was found to vary quite considerably. A comparison of the 3 survival curves shown in Figs. 8.13, 8.14 and 8.15 shows some of the different survival curves obtained. In some experiments, survival curves which almost completely lacked shoulders were obtained, suggesting that the caffeine was responsible for almost completely inhibiting all repair. In other experiments, survival curves with quite marked shoulders were obtained, suggesting that a substantial amount of repair might have occurred. The reasons for this variation are not known. In irradiated exponential phase cells held in AS under anaerobic conditions for 2,5 h, before plating onto caffeine plates, a decrease in survival occurred, but this was not as marked as the decrease in survival obtained when cells were plated directly onto caffeine plates after irradiation. In irradiated exponential phase cells held in RS under aerobic conditions for 2,5 h before plating, a decrease in the amount of LHR occurred, suggesting that in *B. fragilis* some of the repair processes involved in LHR only occur in the cells after they have been plated onto nutrient medium.

8.3.7 THE EFFECTS OF SODIUM ARSENITE ON THE RECOVERY OF CELLS HELD UNDER AEROBIC AND ANAEROBIC CONDITIONS.

Sodium arsenite is known to decrease the survival of repair competent *E.coli* cells. The m.i.c. of sodium arsenite for *B. fragilis* cells was determined in both liquid and solid media and concentrations slightly lower than the m.i.c. were incorporated into plating media

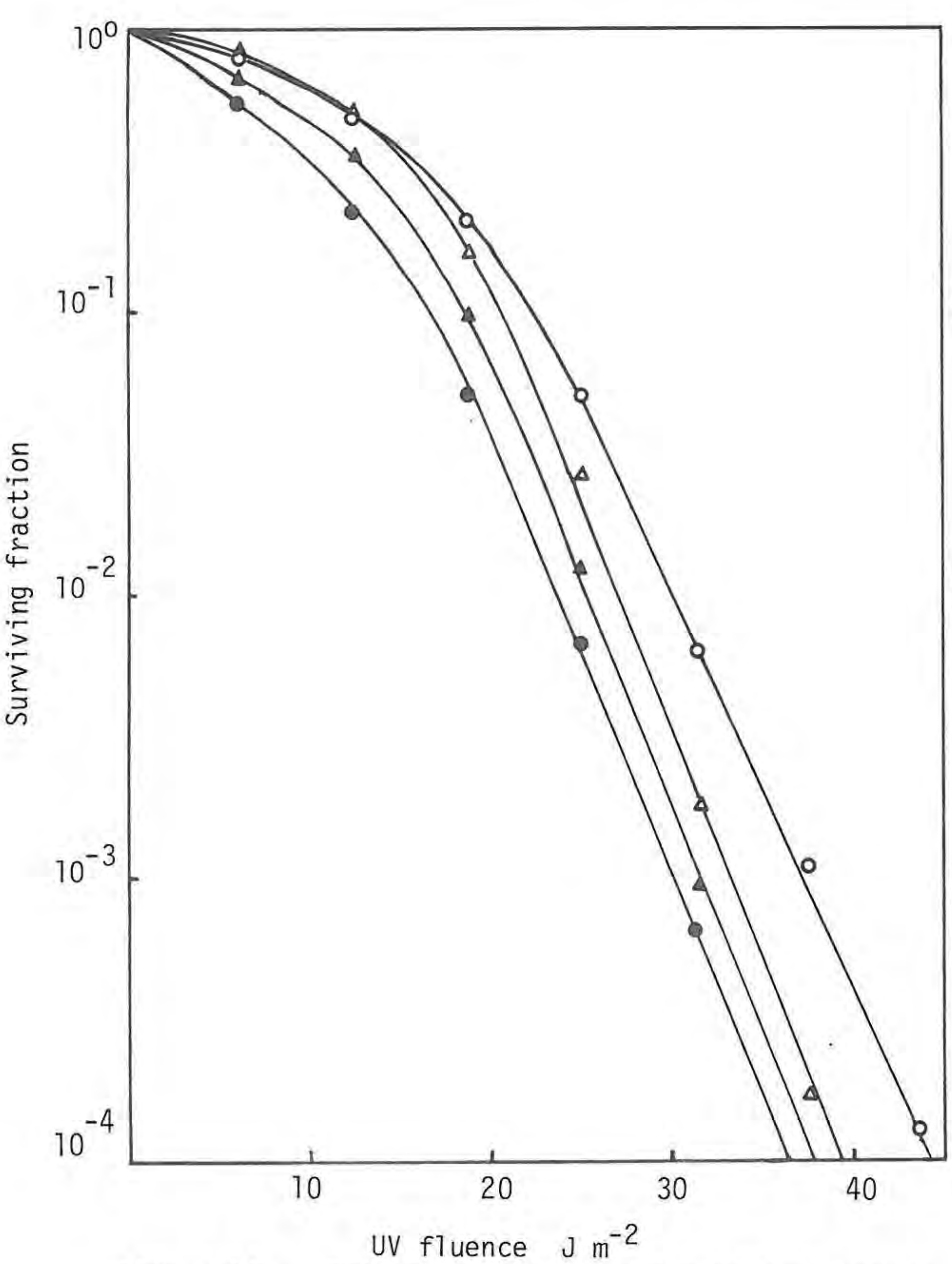


FIG. 8.14: Effect of plating aerobically irradiated exponential phase *B. fragilis* cells onto caffeine plates before and after holding anaerobically in AS for 2,5 h. Cells plated onto BHI at 0 h (o), BHI + caffeine at 0 h (●), BHI at 2,5 h (Δ), and onto BHI + caffeine at 2,5 h (▲).

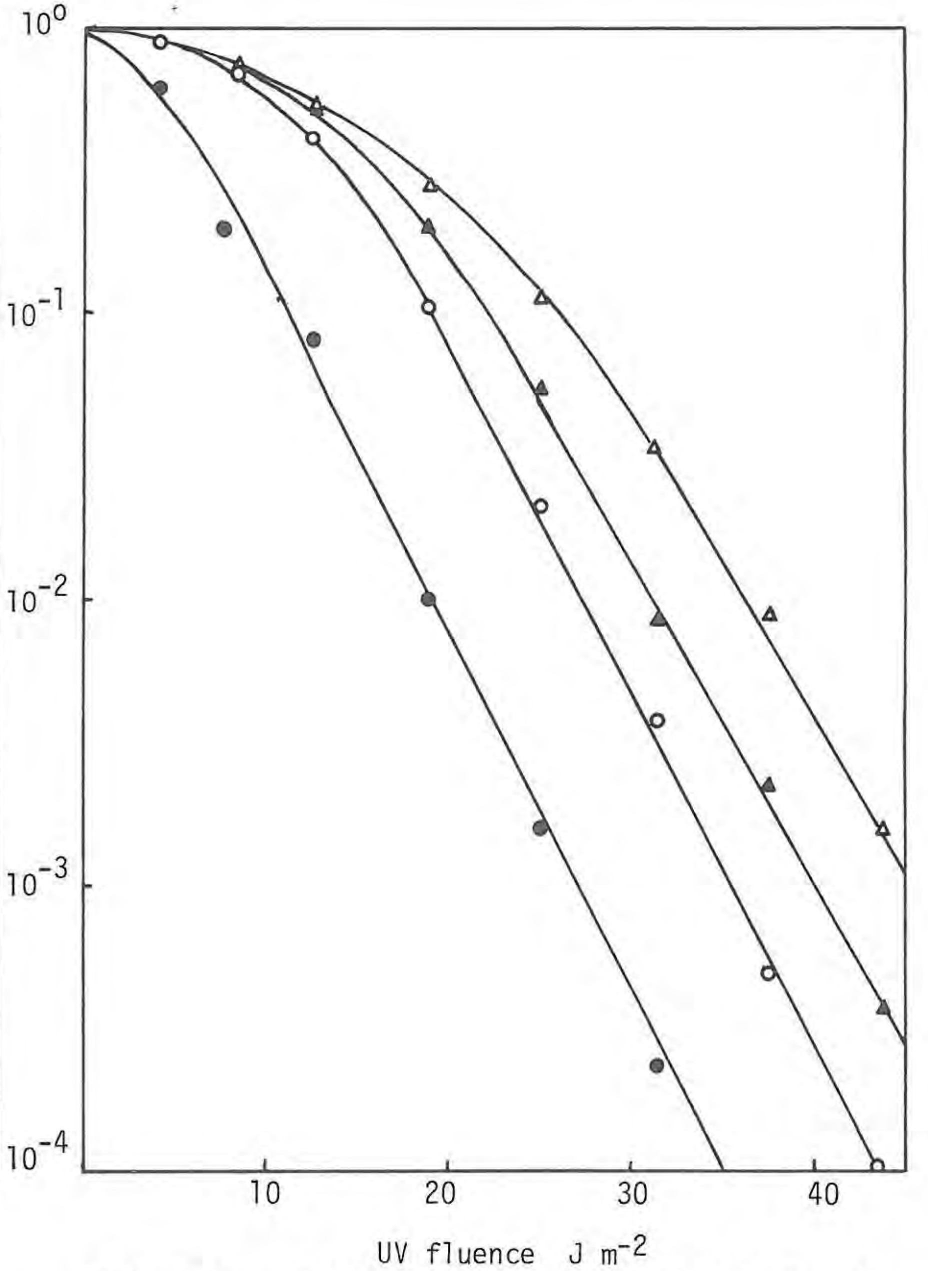


FIG. 8.15: Effect of plating aerobically irradiated exponential phase *B. fragilis* cells onto caffeine plates before and after holding aerobically in RS for 2,5 h. Cells were plated onto BHI at 0 h (o), BHI + caffeine at 0 h (●), BHI at 2,5 h (Δ), and onto BHI + caffeine at 2,5 h (▲).

and anaerobic and aerobic holding buffers (100 μg ml^{-1}). UV irradiated exponential phase cells were held both in AS and RS in the presence of sodium arsenite for 2,5 h under anaerobic and aerobic conditions before plating onto BHI agar plates. No decrease in the amount of LHR occurred in the presence of sodium arsenite under aerobic conditions (Fig. 8.16). The survival of cells held in the presence of arsenite under anaerobic conditions was the same as cells held without arsenite (Fig. 8.16). No decrease in the survival of cells plated directly onto agar plates, containing sodium arsenite, after irradiation was found (Fig. 7.7).

8.3.8 THE EFFECT OF CHLORAMPHENICOL AND NALIDIXIC ACID ON LIQUID HOLDING RECOVERY.

The effect of LHR on the antibiotic, chloramphenicol which inhibits protein synthesis, and nalidixic acid which inhibits DNA synthesis, was investigated. The m.i.c. of the antibiotics were determined and varying concentrations were incorporated into aerobic holding buffers. Time course viability curves for exponential phase cells irradiated with a fluence of 30 J m^{-2} were determined for cells held under aerobic conditions in RS containing increasing concentrations of antibiotics. In cells held in the presence of 1 and 10 μg ml^{-1} chloramphenicol a decrease in the amount of LHR was obtained and a drop in viability during the latter part of the liquid holding period occurred (Fig. 8.17). The m.i.c. for nalidixic acid was 600 μg/ml and it was added to RS at concentrations of 150, 300 and 600 μg ml^{-1}. Nalidixic acid appeared to bring about a retardation of LHR and at concentrations approaching the m.i.c. caused a marked decrease in the amount of LHR (Fig. 8.18).

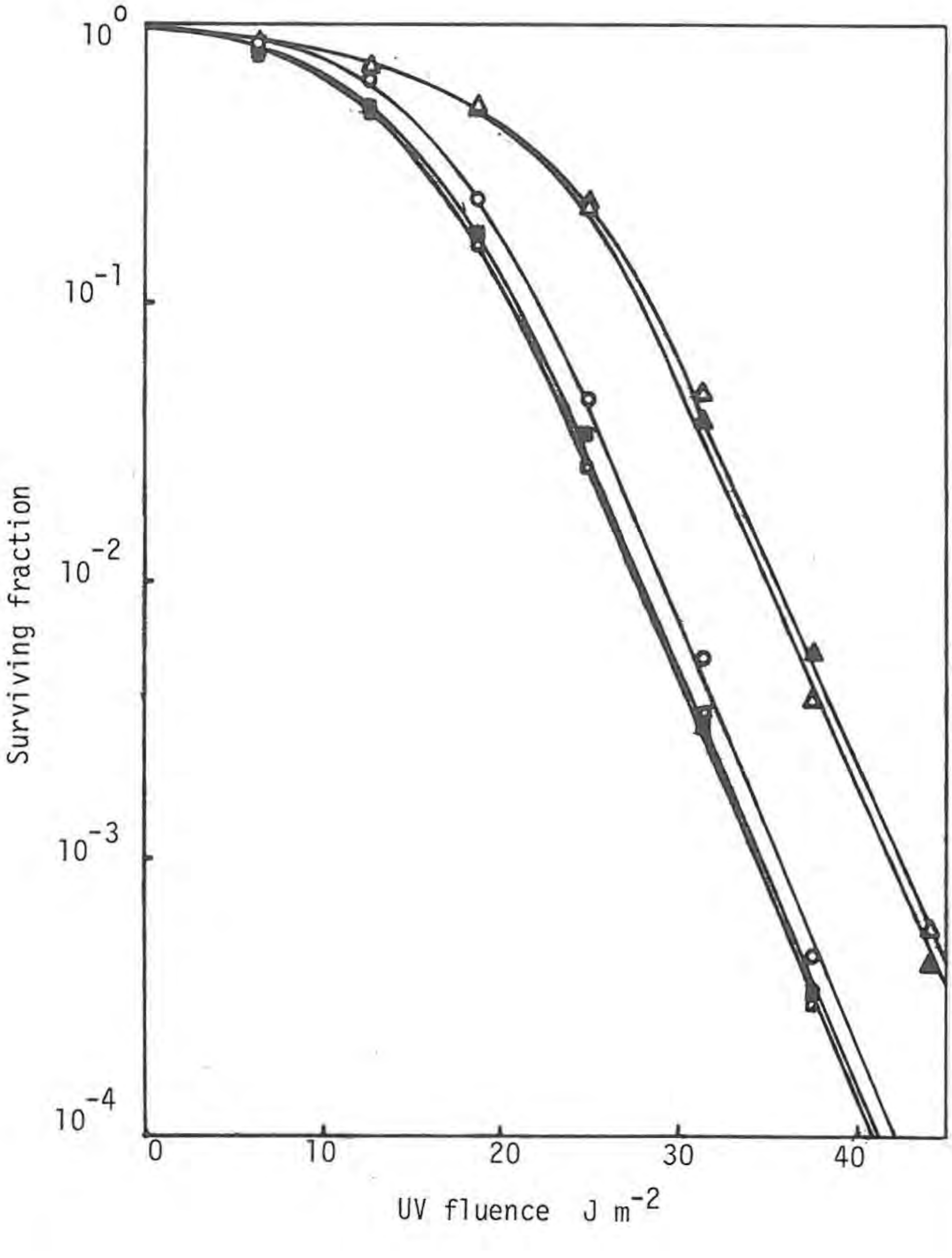


FIG. 8.16: Effect of sodium arsenite on aerobically irradiated exponential phase *B. fragilis* cells held under aerobic and anaerobic conditions for 2,5 h. Cells were plated onto BHI at 0 h (o), held aerobically in RS for 2,5 h (△), aerobically in RS + sodium arsenite for 2,5 h (▲), held anaerobically in AS for 2,5 h (□), anaerobically in AS + sodium arsenite for 2,5 h (■).

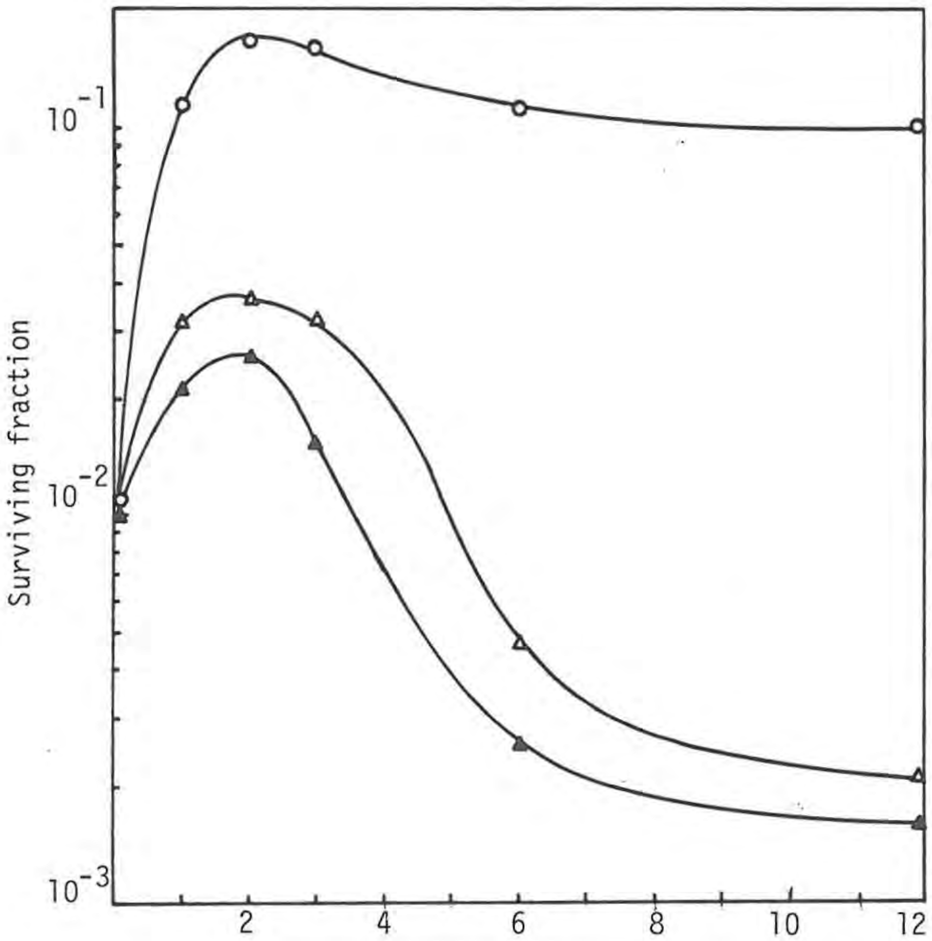


FIG. 8.17: Effect of chloramphenicol on aerobic LHR in *B.fragilis* exponential phase cells irradiated aerobically with 30 J m^{-2} and held aerobically for 0 - 12 h in RS (o), and RS containing 1 (Δ), and 10 g ml^{-1} cml. (▲).

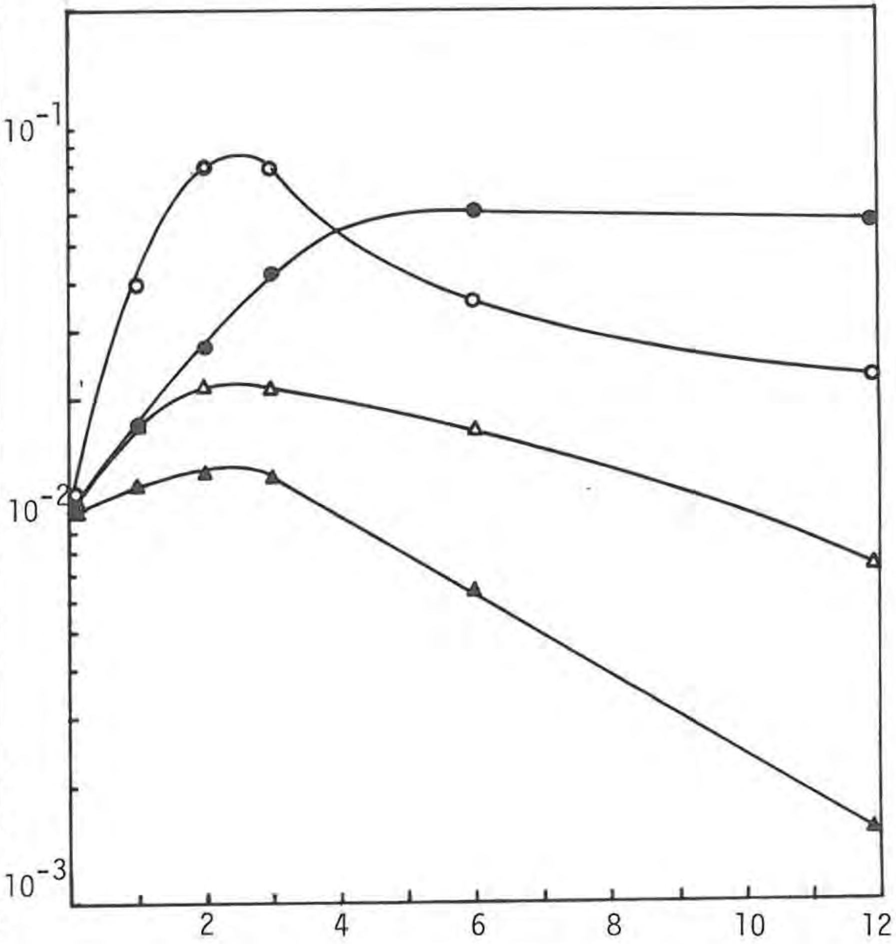


FIG. 8.18: Effect of nalidixic acid on aerobic LHR in *B.fragilis* exponential phase cells irradiated aerobically with 30 J m^{-2} and held aerobically in RS (o) and RS containing 150 (●), 300 (Δ), and 600μg ml^{-1} nal (▲).

8.4 DISCUSSION

8.4.1 GROWTH AND FILAMENT FORMATION IN *B. FRAGILIS* AFTER UV IRRADIATION

Experiments were not carried out to investigate specifically whether growth delay, or inhibition of DNA synthesis occurred in *B. fragilis* cells after irradiation. However, observations suggest that growth delays similar to those reported to occur in other bacterial species might also occur in *B. fragilis* after UV irradiation. These observations included a long lag in growth, delayed colony formation and variations in colony size after UV irradiation. Nothing is known about the effect of UV radiation on DNA replication, or its effect on the bioenergetic and other biosynthetic processes in *B. fragilis* cells.

The formation of long filamentous cells was observed in *B. fragilis* cells subjected to UV irradiation under certain conditions. However, the production of filaments in *B. fragilis* appeared to differ in a number of fundamental ways from those produced in *E.coli* B or mutants of K-12 (*lon*$^-$) after exposure to UV radiation. In *E.coli* the production of filaments has been shown to be an inducible process, similar in many respects to prophage induction and the induction of SOS repair (Witkin, 1976). The specific proteins involved in filament formation in *E.coli* are not known but Witkin (1967) suggested that UV radiation leads to the derepression of a protein which inhibits cell division and septum formation. Filaments are produced as long as the operon remains derepressed. In *E.coli* strains which produce filaments, exposure to low fluences of UV radiation will induce filament formation in the majority of cells, under certain conditions.

However, survival after UV irradiation is not irreversably determined at the time the inducing treatment is terminated, but can be influenced by subsequent manipulation of the media composition, temperature of incubation, and cell density, leading to large variations in the number of survivors. Filamentous growth in *E.coli* is lethal unless cell division is resumed before a certain critical length is reached, and it is the cause of the increased UV sensitivity of *E.coli* B and *lon*$^-$ mutants of K-12. In these strains this increase in sensitivity is seen in the complete absence of any shoulder and a steep drop in the slope of the initial part of the survival curve.

In *B.fragilis* cells, filament production was only observed in a small proportion of the population, and appeared to be fluence dependent. Thus, the production of filaments in *B. fragilis* did not appear to be similar to the inducible process which occurs in *E.coli* . Elongated rods could initially be distinguished from normal vegetative rods after 2 to 3 h incubation following irradiation. These elongated cells continued to increase in length throughout the whole of the growth period, and at the end of the exponential growth phase filaments between 10 and 60 times the length of vegetative rods were observed. The filaments differed from those occurring in *E.coli* strains in that a certain number of septa were always present in the filaments. The number of filaments occurring in cell cultures exposed to the same fluence of UV radiation was found to vary quite considerably. A small percentage of filaments were also observed to occur in unirradiated controls, exposed to the same dilution procedures and holding treatments as the irradiated cells. The proportion of filaments in the population was found to increase as the UV fluence increased, more filaments appeared to be produced after irradiation under aerobic conditions than under anaerobic conditions. In addition, more filaments were observed after liquid holding under aerobic conditions than under anaerobic conditions. It is not known if there is any direct relationship between filament formation and the number of cells surviving after UV irradiation. The shape of the survival curve obtained for *B.fragilis* suggests that the differences in survival in irradiated *B.fragilis* cells under different conditions are not directly related to filament formation as in *E.coli* B and the *lon*$^-$ mutant of K-12. Further studies are required to elucidate the relationship between the production of filaments and the survival of cells after UV irradiation and the relationship of filament formation to the effects of holding cells in buffer under anaerobic and aerobic conditions.

8.4.2 THE EFFECT OF VARIOUS POST-IRRADIATION TREATMENTS ON THE RECOVERY OF *B.FRAGILIS* CELLS.

The LHR process which occurs in *B.fragilis* appears to be unique in that the recovery phenomenon only occurs under aerobic conditions. At first sight this appears to be something of a paradox as the conditions under which recovery occurs appear to be the conditions in which repair processes are operating least efficiently (i.e. in *B.fragilis* exponential phase cells irradiated under aerobic conditions which showed the least resistance to UV radiation). If stationary phase cells were pre-held under aerobic conditions for 2h prior to irradiation, the amount of LHR was increased to give a recovery level similar to the level obtained for exponential phase cells.

A similar situation appears to exist in *E.coli* where LHR can only be observed in cells which have a decreased repair capacity (*E.coli* B or *rec* A mutants of K-12). LHR also occurs to a greater extent in exponential phase cells than in stationary phase cells in *E.coli* (Rupert & Harm, 1966).

In *E.coli* LHR has been shown to be brought about by an excision repair process dependent on functional *uvr* and *pol* A gene products. In *B. fragilis* there is also strong evidence to suggest that LHR is brought about primarily by excision repair. The idea that LHR is brought about by a repair process is supported by the fact that the increase in survival after liquid holding is shown by an increase in the size of the shoulder on the survival curve.

In *B.fragilis*, maximum LHR occurred in non-nutrient buffers, indicating that the recovery process is independent of nutrients and protein synthesis, as in *E.coli*. The increase, both in the rate and amount of LHR was found to be temperature dependent (reaching a maximum at 37°C). This indicates that LHR is an enzyme-mediated process. The ability of caffeine and acriflavine to inhibit LHR indicates that LHR in *B.fragilis* is also brought about by excision repair. Sodium arsenite was found to have no effect on LHR in *B. fragilis* cells. This could indicate that, either sodium arsenite has no effect on a *rec* A type gene product in *B. fragilis*, or that such a gene product is not involved in the recovery process occurring after UV irradiation. *Rec* A processes involving plasmids may be either arsenite sensitive or insensitive in some host cells (Lehrbach *et al.*, 1978). The addition of chloramphenicol to irradiated cells in the holding buffer resulted in a decrease in cell viability. These results suggest that some protein synthesis may be necessary for maximum LHR to occur. This is in conflict with the observation that maximum LHR is obtained under conditions where nutrients are absent. However, there is evidence to suggest that part of the LHR process only occurs after the cells have been plated onto nutrient media, and it is possible that it is this component of the repair process which may be affected by chloromaphenicol. The assessment of the significance of the results obtained with chloramphenicol is difficult because of the extreme sensitivity of *B. fragilis* cells to the antibiotic. After liquid holding, the cells were diluted 10^{-3} or 10^{-4} so that the direct action of chloramphenicol on the plating media after liquid holding appears unlikely.

The addition of nalidixic acid to irradiated cells in the holding solution also caused a decrease in the number of viable cells during LHR, as well as producing a lag in the rate of recovery. When nalidixic acid was added at concentrations equal to the m.i.c., a marked reduction in LHR occurred. At concentrations below this (300 and 150 $\mu g\ ml^{-1}$), the drop in viability was less marked. Nalidixic acid is known to inhibit DNA synthesis in prokaryotic cells, by inhibiting the action of DNA gyrase (Mizuuchi *et al.*, 1978). Scott McDaniel & Hill (1979) have reported that several strains of recombination deficient mutants in *E.coli* show increased rates of viability loss when incubated with nalidixic acid, and they suggest that the nalidixic acid causes damage to DNA either directly or indirectly. Some strains recovered from the effects of naladixic acid at a rate similar to wild-type, but apparently have an imbalance in their metabolism that results in filamentous growth.

Although there appears to be many similarities between LHR occurring in *E.coli* and the LHR which occurs in *B.fragilis*, there are a number of significant differences. The most obvious difference is that LHR in *B. fragilis* only occurs under aerobic conditions and is completely inhibited under anaerobic conditions. A second difference is that LHR in *E.coli* is completely inhibited by the addition of low concentrations of yeast extract to the holding buffer. The mode of action of yeast extract is not known but it appears to bring about increased DNA degradation. The addition of yeast extract, even at concentrations which were 10 fold higher than the concentration which inhibited LHR in *E.coli*, only causes a slight decrease in the LHR of *B. fragilis* cells. A third difference is that in *E.coli*, the whole of the LHR process appears to occur in the liquid holding medium and ceases when cells are plated onto the agar plates. In *B.fragilis* some of the recovery processes appear to continue in cells after they have been plated onto nutrient medium. This further recovery can be inhibited by the presence of caffeine in the plating media. When irradiated cells were given a liquid holding treatment prior to plating onto minimal medium, they showed a marked increase in recovery compared to cells plated onto BHI agar. This indicates that in addition to the repair processes which occur during LHR, further repair processes are carried out after the cells are plated onto nutrient media and these repair processes are enhanced when growth occurs under sub-optimal conditions.

Although LHR was first reported by Roberts & Aldous (1949), the reason that LHR only occurs in repair deficient strains of *E.coli* is not fully understood. Tang & Patrick (1977a) suggested that LHR is presumably not observed in WT K-12 strains because of the presence of a complete repair potential. Once the cells are allowed to grow, any enhancement of survival due to LHR is masked. In a later paper by Tang *et al.* (1979), these workers suggest that LHR depends upon the balance between repair and DNA turnover in cells held under non-growing conditions. They suggest that whatever limits LHR in *E.coli* K-12 must in some way interfere with dimer excision during buffer holding. They have proposed that DNA turnover in regions of DNA strands opposite to a dimer is no longer possible, and attempts to remove the dimer by excision repair results in double-strand breaks which are likely to be lethal.

After preparing various double mutants in combination with *rec* A mutants, Smith (1978b) found that *uvr* D blocked LHR and *rec* F had little effect on LHR. The *lex* A and *rec* B mutations in combination with *rec* A greatly enhanced LHR over that observed in *rec* A alone. His interpretation of these findings is that some excision gaps which are processed by the growth medium dependent pathway may be modified in *rec* A strains, such that they become a substrate for the *pol* A dependent pathway of excision repair. In growth media these excision gaps would be converted to lethal lesions, but in buffer they may be shunted to the *pol* A dependent pathway, be properly repaired and result in enhanced viability. However, he points out that other interpretations of these data are possible.

LHR in *E.coli* and *B. fragilis* appear to be similar in that it only occurs when the full repair capabilities are not present, either because the strain is a mutant which lacks *rec* A dependent repair or when sub-optimal conditions retard or inhibit the full repair processes from occurring. The suggestion by Tang & Patrick (1977a) that LHR is simply masked in the presence of the complete repair potential cannot hold for *B. fragilis* as there is no increase in the survival of cells held under anaerobic conditions even though the full repair capabilities are present under these conditions. The model proposed by Smith (1978b) appears to provide a much better explanation of LHR processes in *B. fragilis*. It seems likely that in *B. fragilis* cells held under aerobic conditions, only *pol* A dependent excision repair occurs (short patch excision repair) and that it is responsible for the increase in viability of cells held aerobically. It is suggested that under these conditions, repair processes in *B. fragilis* can be compared to those occurring in *E.coli* B or *rec* A mutants of K-12. However, under anaerobic conditions, increased repair capability appears to be present, and it is possible that a second mode of repair becomes operational. This second mode of repair resembles *rec* A dependent repair which occurs in *E.coli* K-12 wild-type cells, and in combination with *pol* A dependent excision repair produces a full repair capability under anaerobic conditions. As in *E.coli* K-12, this complete repair potential leads to an absence of LHR in *B. fragilis*, presumably due to the production of lethal lesions either in the liquid holding media or, once growth is resumed when cells are returned to nutrient conditions. Thus it is suggested that in *B. fragilis* , the presence of oxygen inhibits the function of a *rec* A-like gene product which leads to a decrease in the repair capabilities of cells under aerobic conditions. The inhibition of a *rec* A dependent repair pathway under aerobic conditions could account for the marked increase in the sensitivity of cells to UV radiation under aerobic conditions, and explains why LHR only occurs in the presence of oxygen.

CHAPTER IX

REACTIVATION OF *B. FRAGILIS* BACTERIOPHAGES AFTER UV IRRADIATION

9.1 INTRODUCTION

Bacteriophages have proved to be extremely useful and informative biological systems with which to study various aspects of DNA repair and recombination which can occur in the host cell. Studies of phage reactivation phenomena have relied to a large extent on the use of UV radiation as a means of inducing damage in phage DNA. Investigation of the reactivation of UV irradiated phage has led to the elucidation of a number of different repair processes which are involved in this phenomenon. In addition UV radiation is known to cause a number of other physiological effects such as, growth delaying effects, mutagenic effects, and the enhancement of phage recombination. Exposure to UV light is also responsible for inducing phage development in lysogenic bacteria.

Exposure to UV radiation brings about a fairly rapid inactivation of phage particles. The rate at which inactivation occurs is an exponential function of the dose (Adams, 1959). The absorption spectrum for the inactivation of whole phage particles is very similar to that of nucleic acids (Fluke & Pollard, 1949; Putnam, Kozloff & Neil, 1949; Zelle & Hollaender, 1954). The actual rate of inactivation differs quite markedly in different types of phage and the survival of a single phage type can vary under different conditions, and is dependent to a large extent on the properties of the host cell (Luria & Dulbecco, 1949; Adams, 1959). Differences in the sensitivity of different phages can thus be due to both differences in the intrinsic sensitivity of the phage DNA to UV radiation as well as to the degree to which the DNA may undergo subsequent repair in the host cell (Luria & Delbrück, 1942; Cohen & Arbogast, 1950).

After inactivation with high fluences of UV radiation many phages may still retain the ability to kill the host cell, even though the phage is no longer capable of reproducing (Luria & Delbrück, 1942; Cohen & Arbogast, 1950). In phages irradiated with fluences low enough to prevent lethal damage, a considerable increase in the latent period may occur (Luria, 1944).

In addition to the variation in the intrinsic sensitivity of phage DNA to UV radiation, a number of different modes of reactivation are known to occur in the host cells, which are responsible for increasing the survival of irradiated phage (Rupert & Harm, 1966; Swenson, 1976; Adams, 1959; Devoret *et al.*,1975). These reactivation processes may be divided into the following 4 main types according to the nature of the repair processes involved in reactivation of the phage.

(1) Photoreactivation of the irradiated DNA of the infecting phage by the host cell's photoreactivation repair enzyme.

(2) Reactivation of the phage DNA by the process of excision repair either through the action of the host cell repair enzymes as in the case of host cell reactivation (HCR) or by the action of additional phage coded repair enzymes as in the case of v^- gene reactivation which occurs in T4 phage.

(3) Reactivation of the irradiated phage by means of recombination processes, which bring about repair and increased survival. This involves either repair by recombination between two DNA duplexes as in the case of prophage reactivation, and similar repair processes such as multiplicity reactivation and marker rescue (cross-reactivation), or recombination repair involving only one DNA homoduplex as in the case of postreplicative recombination repair.

(4) Reactivation of irradiated phage by repair processes which are induced in the host cell by exposure to UV radiation. UV reactivation or W (Weigle) reactivation, as it is sometimes called, is under the control of the *rec* A gene product in *E.coli* and shows many similarities to the UV inducible system responsible for prophage induction.

9.1.1 PHOTOREACTIVATION

Dulbecco (1950) was the first person to discover that UV irradiated phage could be reactivated by exposure of infected host cells to visible light. Photoreactivation (PR) of irradiated phage DNA is brought about by the same process and uses the same enzyme system that is used to repair damaged host cell DNA. The photoreactivating enzyme is able to bring about the *in situ* repair of DNA by first recognizing and then attaching to cyclobutane dimers. The enzyme substrate complex is then activated by the absorption of light between 320 - 420 nm (Adams, 1959; Hanawalt & Setlow, 1975). The PR enzyme converts the pyrimidine dimers back to monomeric pyrimidine residues and the enzyme is released. Only a proportion of the irradiated phage particles are photoreactivable, and the number is proportional to the UV dose (Adams, 1959).

9.1.2 PHAGE REACTIVATION DEPENDENT ON EXCISION REPAIR

A second type of reactivation of irradiated phage may be brought about by the excision of cyclobutane dimers in the phage DNA, by excision repair processes. In the case of host cell reactivation (HCR) the repair of damaged nucleotide sequences in the phage DNA is dependent entirely on the host cell repair system. In the case of the so called *V* gene reactivation which occurs in the T4 phage the excision repair process requires the action of a phage coded endonuclease and exonuclease.

9.1.2.1 Host Cell Reactivation

Host cell reactivation was first discovered by Garen & Zinder (1955). In repair competent *E.coli* cells the phages T1, T3, T7 and λ can be repaired by the host cell excision repair system, whereas for reasons that are not understood the phages T2, T4, T6 and T5 cannot undergo HCR. They are also more sensitive to UV radiation in repair competent cells (Swenson, 1976). HCR of temperate and semi-temperate phages has been shown to exist in all the vavious bacterial species where it has been sought (Devoret *et al.*, 1975).

Only *E.coli* cells which possess an intact excision repair system can carry out HCR and this depends on the functions of at least four genes, *uvr* A, *uvr* B, *uvr* C, and *uvr* D, (Devoret *et al.*, 1975). Boyle & Setlow (1970) were the first to demonstrate that HCR is due to pyrimidine dimer excision which is only carried out by cell enzymes. In addition to the *uvr* gene products, Monk *et al.*, (1971) demonstrated that functional *pol* A1 gene product was necessary for HCR. Inhibitors which are known to inhibit excision repair also inhibit HCR. Examples of such inhibitors of HCR are acriflavine (Feiner & Hill, 1963) and caffeine (Metzger, 1964; Sauerbier, 1964).

HCR has an efficiency of about 85% (Harm, 1963) so that it is by far the most efficient repair process leading to phage reactivation. The repair is accurate and no phage mutation results from the action of the repair process (Devoret *et al.*, 1975).

Competition experiments have shown that the host cell enzyme preferentially exises dimers from host cell DNA and that the rate of dimer excision from bacterial cell DNA is 30 times faster than from phage DNA (Boyle & Setlow, 1970).

Harm (1974) has put forward evidence to suggest that in repair competent *E.coli* B/r there may be two modes of excision repair. The first mode being very efficient and capable of repairing most of the lesions in the DNA. When this mode is inhibited from operating a second less efficient mode becomes operational. This second mode is not affected by caffeine or acriflavine and is therefore not thought to be due to normal HCR (excision repair).

In host cells unable to carry out HCR (HCR^- cells), Boyle & Setlow (1970) observed that 10 - 20% of the phage dimers may be removed and they attribute their removal to recombination repair. The way in which the excision repair processes and the recombination repair processes interact in repairing damaged phage DNA was investigated by Radman *et al.*, (1970) using λ phage. From the results of their investigations they concluded that at low fluences, most of the repair of UV irradiated DNA is carried out by the recombination repair system, and at high fluences most of the repair occurs through the HCR process. Radman *et al.*, (1970) also showed, by using temperature sensitive phage mutants in which DNA synthesis could be inhibited at 42^oC, that excision repair enzymes work best in the absence of DNA replication. A model was proposed by Radman *et al.*, (1970) in which they suggest that the extent of repair by the two repair systems is controlled by the degree to which DNA synthesis is inhibited by UV irradiation. Up to a certain 'critical' fluence little inhibition occurs and because semiconservative replication is taking place, recombination repair is dominant. Above the critical fluence DNA synthesis is inhibited, allowing excision repair to take place. They also suggested that the timing of the resumption of DNA replication is important for the maximum efficiency of repair to take place.

9.1.2.2 *V* gene reactivation in bacteriophage T4.

Luria (1947) made the observation that T4 phage was about twice as resistant to UV radiation as the phages T2 and T6. It was originally thought that the increased resistance in T4 was due to the absence of a locus (the U^+ allele). However, later work by Harm, (1961) showed that the *U* gene actually coded for a function or enzyme which was responsible for the increased survival of the T4 phage. This discovery lead to the *V* gene with the allelic state of V^+ for the T4 phage and V^- as the mutant or T2 state.

Setlow & Carrier (1966, 1968) demonstrated that the excision of dimers from irradiated T4 DNA was dependent on the *V* gene product. Later Friedberg & King (1969) and Yasuda & Sekiguchi (1970) showed that the *V* gene codes for an endonuclease activity which is expressed in T4 infected cells, and has been called endonuclease V of T4 or T4 UV endonuclease. Later work by Sekiguchi *et al.* (1975) showed that the *V* gene is a structural rather than a regulator gene for the endonuclease. The enzyme is induced early after infection (Freidberg & King, 1971) and attacks double stranded UV irradiated DNA, making single stranded endonucleolytic excisions by causing phosphodiester bond breaks on the 5' side of the dimer. The T4 UV endonuclease does not excise thymine dimers from UV irradiated DNA (Friedberg & King, 1971) but Sekiguchi *et al.*, (1975) and Freidberg *et al.*, (1974) have shown that the phages T2, T3, T4 and T6 also code for exonuclease activity that can excise thymine dimers from already nicked DNA.

Harm (1973) has shown that caffeine and acriflavine which are inhibitors of HCR, have little or no effect on *V* gene reactivation.

It is not known why T4 phage which can undergo *V* gene reactivation or the T2, T5 and T6 phages of *E.coli* do not undergo HCR. Although the T 'even' phages do have modified nucleotides which could be implicated, these modifications do not occur in the T5 phage (Friedberg, 1975).

9.1.3 PHAGE REACTIVATION DEPENDENT ON RECOMBINATION REPAIR

Two types of recombination repair appear to be involved in phage reactivation phenomena (Devoret *et al.*, 1975).

The first type of recombination repair involved in phage reactivation occurs when only one DNA homoduplex is involved in the recombination repair process and recombination can only take place after DNA replication has occurred. This repair process was first discovered by Howard-Flanders *et al.*, (1968) and has been termed postreplicative recombination repair. It is thought to be important in bringing about repair in phages exposed to low fluences of UV radiation (Boyle & Setlow, 1970).

A second type of recombination repair involving two DNA duplexes has been shown to occur in prophage reactivation (Jacob & Wollman, 1953; Yamamoto, 1967) and in similar repair processes such as multiplicity reactivation (Luria, 1947) and marker rescue or cross-reactivation (Doermann, 1961). Although all three processes show similarities they differ in that in prophage reactivation only the infecting phage DNA is irradiated whereas in multiplicity reactivation all the phage DNA molecules are irradiated. In marker rescue only the DNA containing the rescued marker is irradiated. In principle there is no need for any extensive DNA synthesis to occur in this type of recombination repair as the two strands of DNA which are involved in the recombination process are already present in the cell. There is evidence to support the idea that these three types of reactivation occur as pre-replicative recombination processes. Baker *et al.*, (1971) and Jacob & Wollman (1955) showed that recombination appears to be triggered by the presence of pyrimidine dimers in the phage DNA and that UV induced recombination occurs before DNA replication takes place. Howard-Flanders & Lin (1973) showed that marker rescue between phage and prophage can occur when the replication of phage DNA is blocked by immunity. It has also been suggested that the growth delay which occurs in irradiated phages is due to the inhibition of DNA replication and this delay allows for the pairing and recombination of chromosomes to occur before replication takes place (Devoret *et al.*, 1975). However, a number of workers have shown that recombination is always associated with DNA synthesis (see review by Radding, 1973) and the term pre-replicative recombination repair should be used with caution.

9.1.4 PROPHAGE REACTIVATION

Prophage reactivation is brought about by recombination between the homologous DNA of UV damaged infecting phage and an intact resident prophage and leads to an increased survival of the infecting phage (Chase, 1964; Devoret & Coquerelle, 1966; Yamamoto, 1967; Hart & Ellison, 1970; George & Devoret, 1971). It only occurs when the prophage in the host cell is noninducible and was first discovered in phage λ by Jacob & Wollman (1953) and later in phage P2 by Chase (1964) and in phage P22 by Yamamoto (1967).

Prophage reactivation is essentially a repair process which is able to bypass pyrimidine dimers which have not been excised. About 50% of the unexcised dimers may be repaired by this process. This value remains constant and is independent of whether the host cell is able to excise dimers or not (Devoret *et al.*, 1975). In an excision repair competent host (HCR^+) 85% of the dimers can be repaired by HCR and 50% of the remaining dimers can be repaired by prophage reactivation (92.5%). In excision repair deficient hosts (HCR^-) 50% of the total number of dimers can be repaired by prophage reactivation (Devoret *et al.*, 1975).

The nature of prophage reactivation has been investigated using both recombination deficient host cells (*rec* A, *rec* B, *rec* C) as well as recombination deficient λ phage mutants (*red* and *int* mutants). Blanco & Devoret (1973) showed that in *rec* A^- cells, reactivation had an efficiency of 13% compared with 53% reactivation in rec A^+ cells. They concluded that prophage reactivation depends to a large extent but not entirely on the presence of the *rec* A gene product. They also showed that the small amount of reactivation in *rec* A^- cells was dependent on the *red* recombination gene of the λ phage but was not affected by the λ *int* gene.

Prophage reactivation has been shown to be independent of *rec* B, *rec* C and *lex* function as well as *uvr* function (Hart & Ellison, 1970; George & Devoret, 1971; Blanco & Devoret, 1973). Prophage reactivation does not cause mutations despite the high rate of recombination between the irradiated infecting phage and the prophage in the unirradiated cell (Blanco & Devoret, 1973).

9.1.5 MULTIPLICITY REACTIVATION

When two or more UV inactivated phage particles infect the same host cell, they will often co-operate in such a way as to produce viable progeny. This phenomenon is known as multiplicity reactivation (MR) and it was first discovered by Luria (1947) and investigated in more detail by Luria & Dulbecco (1949) and Dulbecco (1952). MR appears to be quite common in phages and has been shown to occur in the T even phages, T5, and λ phages (Kellenberger & Weigle, 1958). MR has also been shown to occur in T1 phage under conditions where no HCR occurred (Tessman & Ozaki, 1959). Harm (1966) showed that MR was increased in λ phage in host cells which were unable to carry out HCR (*uvr*$^-$)

Luria & Dulbecco (1949) were the first to suggest that recombination might play a role in MR. UV irradiation was found to increase the frequency of genetic recombination in phage λ (Jacobs & Wollman, 1955) and in T4 (Hershey *et al.*, 1958). Epstein (1958) showed that the frequency of recombination was much higher in multiplicity reactivated complexes. Huskey (1969) using both *rec*$^-$ host cells and *rec*$^-$ λ phage showed that MR occurred when either the host or the bacteriophage were *rec*$^+$, but when both the host and the phage were *rec*$^-$ there was no MR. His findings indicate that both the bacterial and the phage recombination systems are involved in the MR of λ phage. Symonds (1975) also showed that T4 phage mutants which show a reduced potential for recombination also show a reduced level of MR.

9.1.6 MARKER RESCUE (CROSS REACTIVATION)

If a UV inactivated phage carrying one or several genetic markers, co-infects a host cell with an unirradiated, unmarked particle of the same species, some of the viable progeny particles may contain genetic markers of the UV irradiated phage. This phenomenon has been termed marker rescue or cross-reactivation (Luria & Dulbecco, 1949) and it shows clearly that genetic recombination with UV inactivated particles is possible, even if the UV treatment was extremely severe (Doermann, 1961). Marker rescue therefore appears to have some similarity to multiplicity reactivation.

9.1.7 *X* GENE REACTIVATION

In addition to an excision repair system T4 phage also codes for a second mode of DNA repair. This system requires the *x* gene (Harm, 1963b)and the *y* gene (Boyle & Symonds, 1969). These genes appear to be involved in a pathway of repair which is independent of the excision repair pathway and which in some respects appears to resemble the postreplication repair process which occurs in bacteria (Mortelmans & Friedberg, 1972; Symonds *et al.*, 1973). However, very little is known about this mode of repair. In addition to affecting the recombination processes in T4 the *x* gene has also been shown to affect the DNA synthesizing system of T4 (Sekiguchi *et al.*, 1975).

9.1.8 UV REACTIVATION

The survival of UV irradiated phage λ increases when the host cell has been exposed to low UV fluences before infection. This phenomenon was discovered by Weigle (1953) and is known as UV reactivation (UVR) (it is also sometimes called Weigle reactivation or W reactivation), and is accompanied by an increase in the rate of mutation in the reactivated phage. Although it has been most intensively studied in phage λ UVR is also known to occur in a number of other phages including T1 and T3 but not T2 or T5. The repair efficiency can be as high as 50%, however, UVR acts on DNA lesions which remain after pyrimidine dimer excision has occurred, so that in a host cell in which excision repair can occur, UVR only accounts for the repair of about 8% of the total lesions. UVR of the phage has been shown to depend entirely on the host cell processes and phage genes such as *red* A, *red* B and *gam* which promote or allow recombination in λ are not involved (Devoret *et al.*, 1975). Shimada *et al.* (1968)found that UVR requires protein synthesis in the host cell. The only mutations in the host cell which have been found to inhibit UVR are *rec* A and *lex* mutations, other mutations such as the *uvr*, *pol* A, *rec* B and *rec* C which are involved in other repair processes in the host cell, do not affect UVR (Howard-Flanders & Boyce, 1966; Ogawa *et al.*, 1968; Miura & Tomizawa, 1968; Defais *et al.*,1971; Radman & Devoret, 1971).

Since the discovery of UVR 3 hypotheses have been advanced to account for the way in which enhanced phage survival comes about. Harm (1963b) suggested that UVR was brought about by the enhancement of the HCR process (in the irradiated cell) caused by irradiation of the host cell, which led to more efficient repair of the irradiated phage DNA. The involvement of pyrimidine dimer excision in UVR was controvertial for a long time but it has been shown that UV reactivation is not the result of enhanced dimer excision as it occurs in *uvr*$^-$ cells in which there is no pyrimidine dimer excision (Defais *et al.*, 1971; Radman & Devoret, 1971). Genetic recombination as the mechanism for UVR has been postulated by a number of authors (Garen & Zinder, 1955; Kellenberger & Weigle, 1958; Weigle, 1966), and Hart & Ellison (1970) extended this hypothesis to suggest that UVR is analogous to prophage reactivation. Experiments done by Blanco & Devoret (1973) to compare the extent of UVR in normal hosts and hosts containing integrated, but non-inducible prophages showed that slight increases in UVR occurred in prophage containing cells, but that less recombination between prophage and infecting phage was found in irradiated cells than in unirradiated cells. Thus recombination of the irradiated phage does not appear to be involved in UVR. UVR has also been found to occur in the single stranded DNA phages S13 (Tessman & Ozaki, 1960) and ϕR (Ono & Shimazu, 1966) indicating that post-replication recombination repair cannot be responsible for UVR as this process can only operate on double stranded DNA. In addition to UVR and UV mutagenesis of phage λ the *rec* A and *lex* A genes are also known to be essential for a number of other UV dependant phenomena, such as the induction of prophage, mutagenesis in *E.coli*, and a number of other physiological effects in irradiated cells. This led Defais *et al.* (1971) to suggest that common pathways might be involved in these phenomena. Both lysogenic induction and UVR can be obtained indirectly by conjugation with irradiated F^+ or F' prime doners (Borek & Ryan, 1958; Devoret & George, 1967; George & Devoret, 1971; George *et al.*, 1974). The level of mutation in indirect UVR is the same as that produced by direct UVR. Both indirect UVR and indirect prophage induction have been shown to result from the transfer of UV damaged DNA to the host cell (Devoret *et al.*, 1975). The UV damaged DNA apparently acts to induce error prone repair processes and other inducible physiological effects, which require the presence of functional *rec* A, and *lex* gene products in the cell. Prophage induction and UVR show many similarities to inducible repair processes in *E.coli*. The SOS repair hypothesis, first put forward by Radman (1974) suggests that an inducible repair process acts on both UV-damaged phage and bacterial DNA in such a way that a common biochemical pathway relates, UV mutagenesis, UV lysogenic induction, UV reactivation, delayed cell division, aberrant reinitiation of DNA replication and respiration shut off. The hypothesis is based on the pleiotrophic effects of *rec* A and *lex* mutations and the model is discussed further in Chapter 10.

9.2 METHODS

9.2.1 UV IRRADIATION PROCEDURES

The phage strains and the methods used to propagate and assay them have been described in 4.2.2 - 4.2.3 The radiation source and the dosimetry used for irradiating *B.fragilis* phages and their host cells have also been described previously (7.2.3). Phage lysates were diluted in T2 buffer to give 10^8 p.f.u. ml^{-1}. Phage suspensions were irradiated in open glass petri dishes and were stirred or shaken during irradiation and the irradiated phage suspensions were then stored at 4°C. Sufficient phage was prepared so that a large number of aliquots could be obtained from each sample allowing for a number of experiments to be performed on the same sample. No loss in phage viability occurred in these suspensions during the 6 month period during which these experiments were conducted. In experiments performed to investigate UVR in *B. fragilis*, host cell suspensions were either irradiated with a fixed fluence of 25 J m^{-2} or with increasing fluences of UV radiation, according to the method described in (7.2.3).

9.2.2 METHODS USED TO DETERMINE THE SURVIVING FRACTION OF IRRADIATED PHAGE UNDER DIFFERENT CONDITIONS.

The surviving fraction of irradiated phage was determined using the soft agar overlay technique (Adams, 1959). Irradiated phage suspensions on phage host cell complexes were diluted to give suitable plaque counts and added to a tube of soft agar (2,5 ml of 0,75% BHI agar). Approximately 5 x 10^8 of late exponential phase cells (OD 0,8) were added as the indicator. The overlay plates were incubated anaerobically for 24 h at 37°C to allow for plaque formation. A minimum of 3 plaque counts were done for each dilution and the mean of the counts was used to calculate the surviving fraction of the p.f.u. The effect of caffeine on the survival of irradiated phage was determined by incorporating 1 mg ml^{-1} caffeine into BHI agar plates which were used in the overlay technique. Host cells irradiated with fixed or increasing fluences of UV radiation were used in place of unirradiated indicator cells in the experiments designed to investigate UVR in *B. fragilis* phages.

In the experiments investigating MR, samples of phage irradiated with increasing fluences of UV radiation were allowed to absorb onto exponential phase *B. fragilis* cells at a m.o.i. of much less than 1 to give monocomplexes and at a m.o.i. of greater than 1 to give multicomplexes. The number of p.f.u. in each sample of irradiated phage was first determined and known numbers of host cells (pre-determined by plate counts) were diluted to give a m.o.i. of *c.* 0,1 or 0,01 in each monocomplex and *c.*5 in each multicomplex. Monocomplexes of infected bacteria were assayed by the soft agar technique. With the multicomplexes of infected cells, the cells were first washed by centrifugation to remove any free phage particles before being assayed. Uninfected exponential phase cells were used as the indicator cells, and the overlay plates were incubated under anaerobic conditions for 24 h at $37^{o}C$ and the number of infective centres were counted. In the experiments done to investigate MR under anaerobic conditions, the entire procedure was carried out in an anaerobic glove box.

9.2.3 METHOD USED TO SCREEN FOR HCR$^-$ MUTANTS IN *B. FRAGILIS*

A modification of the method of Howard-Flanders & Theriot (1962) was used to screen for radiosensitive HCR$^-$ mutants in *B. fragilis*. Mutations were induced in the bacteria by exposure to ethane methanesulfonate (EMS) using a modification of the technique described by Van Tassell & Wilkins (1978). Volumes (1 ml) of exponential or stationary phase *B. fragilis* cells were inoculated into a 10 ml volume of BHI broth, and incubated at 37^{o}C and when mid-log phase was reached (OD 0,5), EMS was added aseptically to the culture to give a final concentration of 2,5% (v/v). The culture was then reincubated at 37^{o}C for a further 30 m and then either diluted and plated immediately onto BHI plates, or washed twice by centrifuging in buffer before resuspending in BHI broth at an OD 0,2. The cells were then reincubated at 37^{o}C overnight (15 h) and plated onto BHI plates at a dilution which would produce between 500 to 1000 colonies per plate. The plates were then incubated under anaerobic conditions at 37^{o}C for 4, 6 or 8 h at which time the colonies were still not large enough to be visible. FC1 or FC2 phage which had been exposed to a fluence of 180 and 240 J m^{-2} respectively, were added to 2,5 ml of soft agar at a concentration of 5 x 10^8 ml^{-1}. The soft agar was mixed and very carefully added to the surface of the inoculated plate using a pipette. The plates were then incubated for a further 24 to 48 h. Surviving colonies were picked and streaked onto BHI agar and single colonies isolated. These isolates were then tested for decreased sensitivity to UV radiation and ability to show plaque formation with the two phages.

9.2.4 METHOD USED TO OBTAIN PHAGE MUTANTS

The substitution of the base analogue 5-bromouracil during DNA synthesis is known to bring about mutations, and has been used extensively to obtain phage mutants (Benzer & Freese, 1958). A modification of the method described by Symonds (1968) for obtaining phage mutagenesis with 5-bromouracil was used. *B. fragilis* cells were grown overnight in minimal liquid medium and 0,5 ml was inoculated into a sulphanilomide minimal medium containing 50 μg ml^{-1} 5-bromouracil. The medium was incubated at 37^{o}C for 4 h and 0,1 ml of FC2 phage (10^{8} p.f.u. ml^{-1}) was added under anaerobic conditions. The culture was then incubated for a further 4 h prior to the addition of 10% chloroform (v/v). The culture was shaken for 10 s and allowed to stand at room temperature for 15 m. The lysate was diluted and assayed by the soft agar technique. The assay plates were incubated overnight at 37^{o}C under anaerobic conditions and any plaques showing abnormal morphology were picked using sterile tooth picks and re-assayed for the mutant plaque morphology.

9.3 RESULTS

9.3.1 THE INACTIVATION OF *B. FRAGILIS* PHAGES BY UV RADIATION

The rate of UV inactivation of 12 *B. fragilis* phages was determined. Six of the phages produced moderate or large clear plaques on *B. fragilis* BSP1 (FC 1,2,3,4,5,6) and the other six produced small turbid plaques (FT 1,2,3,4,5,6). The phages which produced turbid plaques proved difficult to work with due to difficulty in obtaining accurate plaque counts because of their indistinct nature. However, in all 6 phages, the survival was found to be an approximate exponential function of the UV fluence (Fig. 9.1). These phages showed a 1,55 fold variation in their sensitivity to UV radiation at 1% survival, with FT1 and 2 being most resistant to UV radiation, FT 3,4 and 5 being least resistant, and FT 6 being intermediate (Fig. 9.1). In the phages showing clear plaque morphology, 5 of the 6 phages did not show simple exponential kinetics of inactivation, but had convex survival curves with a shoulder of varying size (Fig. 9.2). These phages showed a greater range in their sensitivity to UV radiation (a 1,85 fold range at 1% survival) and most of them were more sensitive to UV radiation than the phages which produced turbid plaques. When compared with the UV radiation sensitivity of *E.coli* T phages, all 12 of the *B. fragilis* phages showed a marked increase in resistance to UV radiation (Fig. 9.3).

The phenomenon involving variations in e.o.p. when phages were plated onto host cells of different ages, (See 4.3.3) proved to be a major problem when investigating the inactivation of *B. fragilis* phages by UV radiation. When aliquots of the same irradiated phage samples were plated with indicator cells of different ages, survival curves with differing slopes and differing shapes were obtained. However, when the surviving fraction for each curve was calculated using the same base (e.g. calculated relative to the survival levels obtained with late log phase cells), the slopes and the shapes were quite similar. Although the level of survival of the phage varied over a 10 fold level when plated on indicator cells which were 8 h and 24 h old respectively, the survival curves obtained were found to be approximately parallel (Fig. 9.4). The variation in both the slope and the shape of the

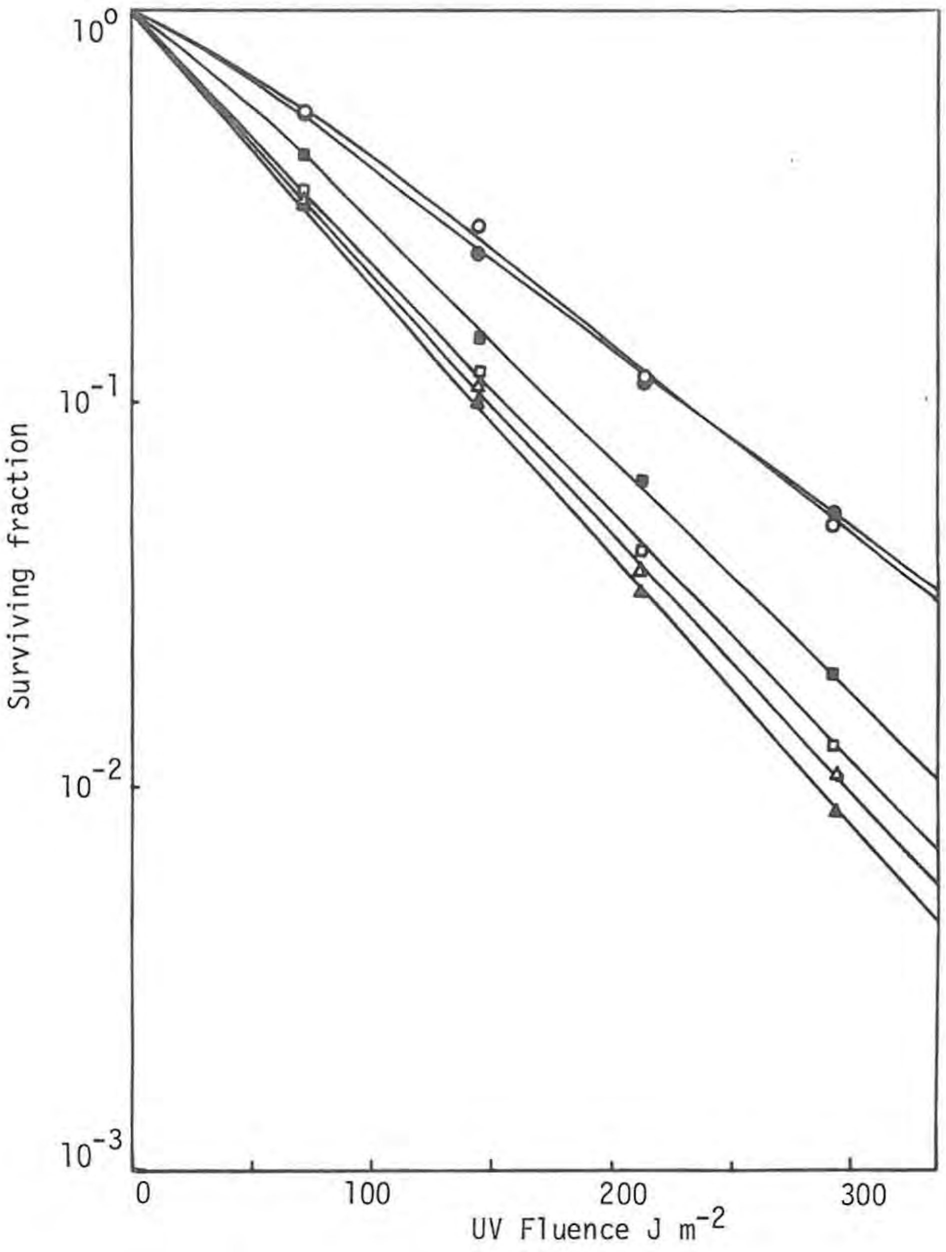


FIG. 9.1: Inactivation of *B.fragilis* phages FT1 (o), FT2 (●), FT3 (Δ), FT4 (▲), FT5 (□), FT6 (■), (turbid plaques) by UV radiation.

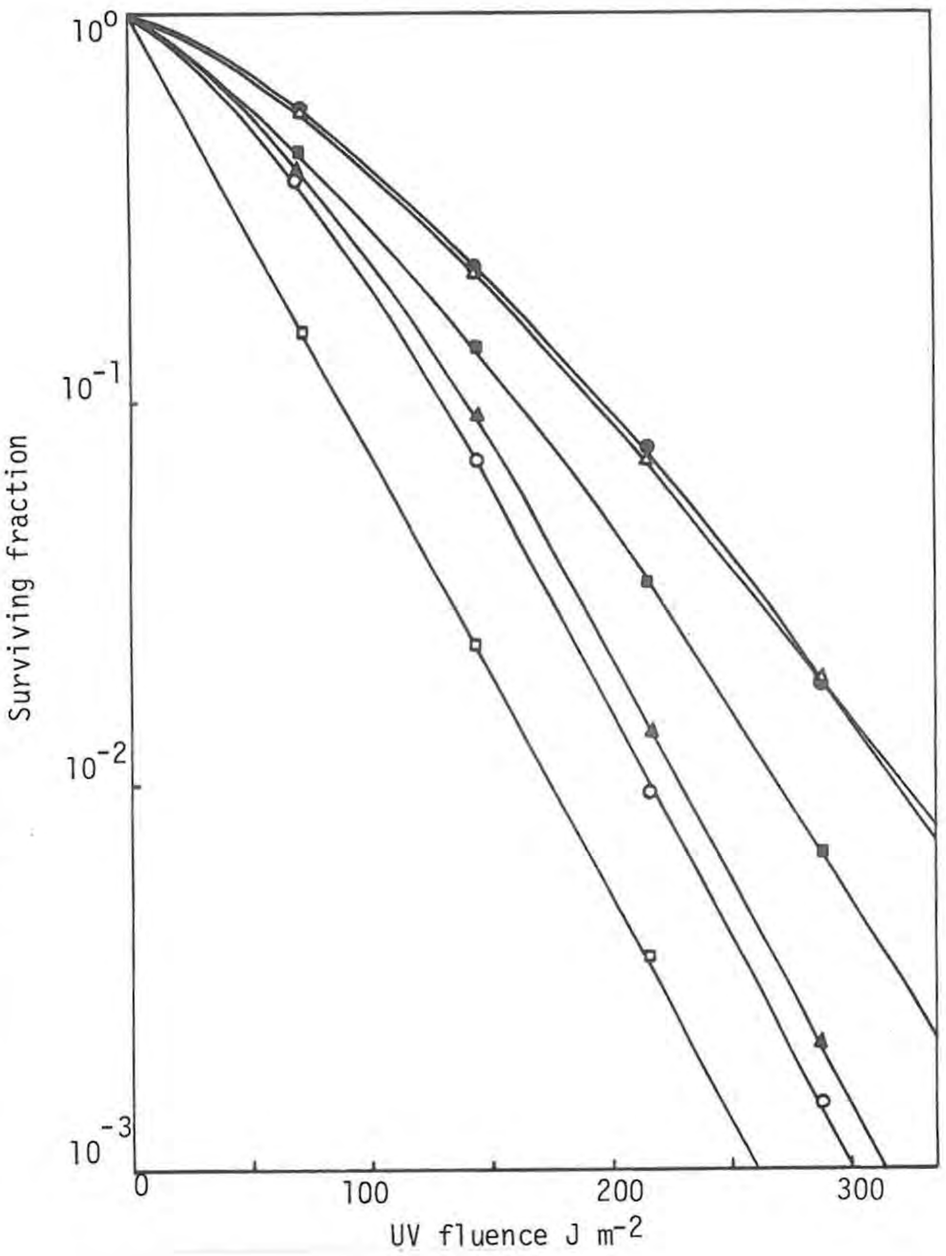


FIG.9.2: Inactivation of *B.fragilis* phages FC1 (o), FC2 (●), FC3 (Δ), FC4 (▲), FC5 (□), FC6 (■), (clear plaques) by UV radiation.

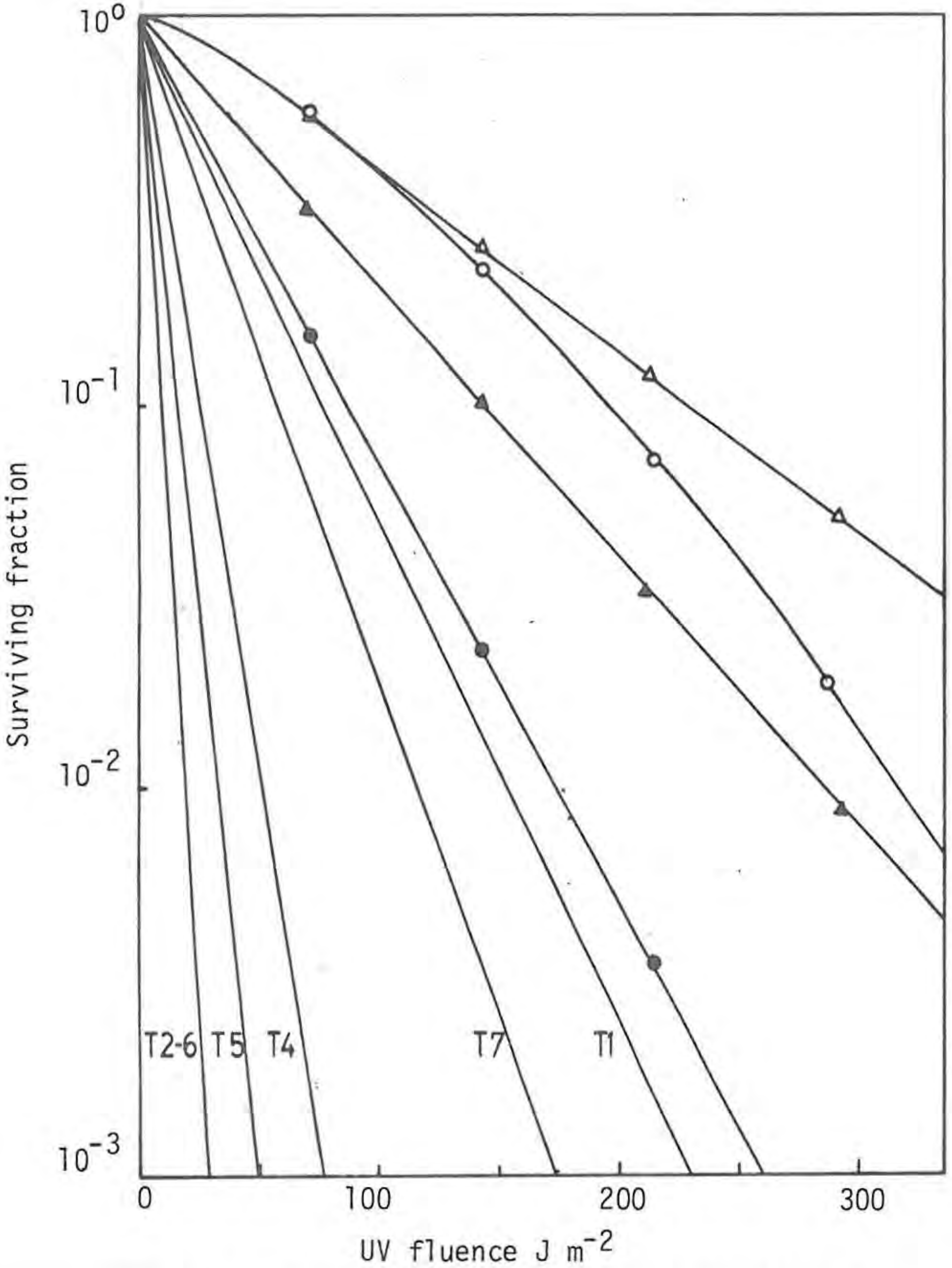


FIG. 9.3: Comparison of UV inactivation of *B.fragilis* phages FC2 (o), FC5 (●), FT1 (△), and FT4 (▲) (most sensitive o△ , least sensitive ●▲) and *E.coli* T1 - T7 phages.

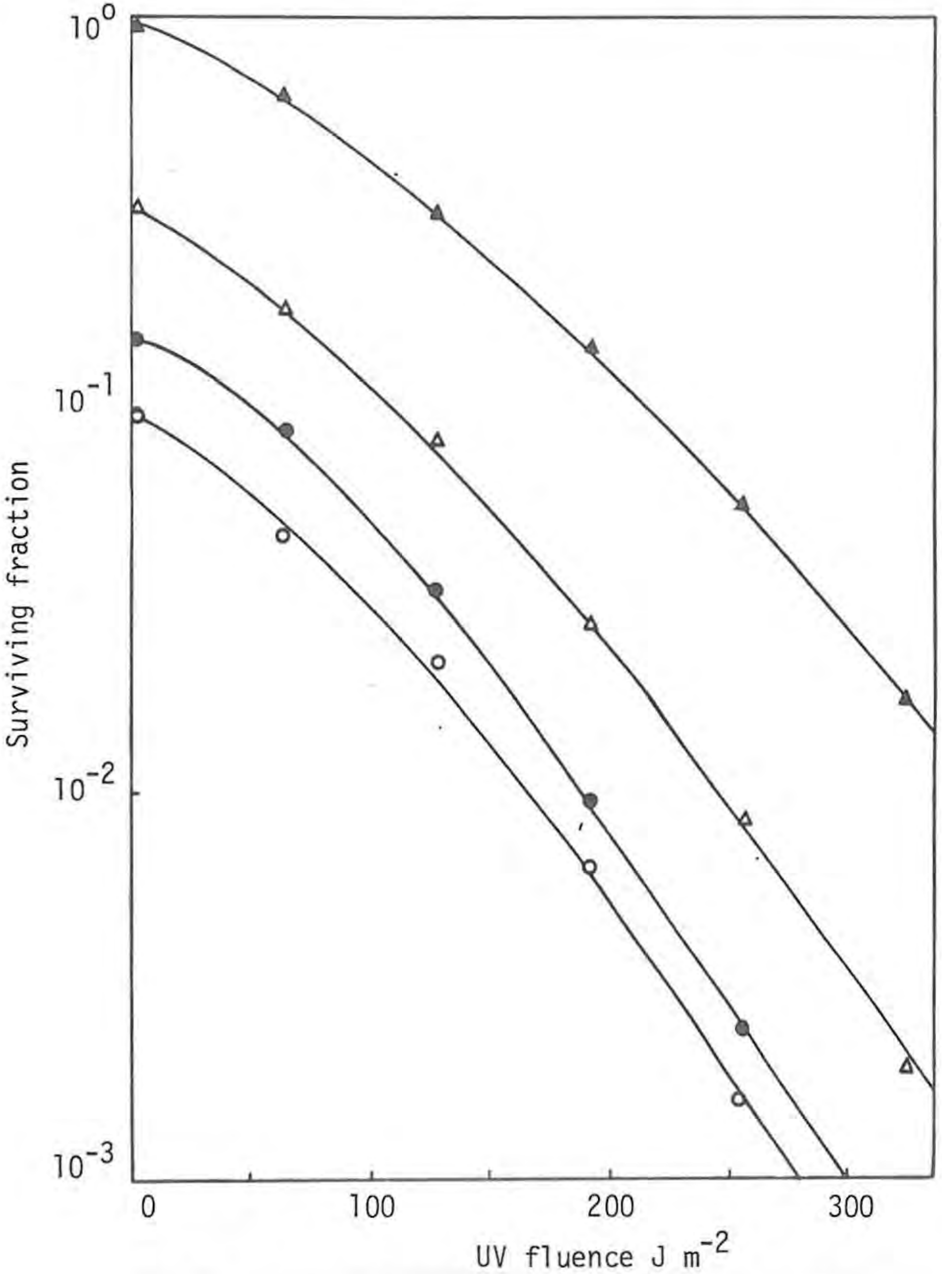


FIG. 9.4: Effect of the age of *B.fragilis* cells (increasing e.o.p.) on the survival of UV irradiated FC2 phage. FC2 phage was assayed on *B.fragilis* cells incubated for 8 (o), 10 (●), 15 (△), and 24 h (▲).

survival curves obtained for the same phage meant that it was only possible to compare survival curves of phages which had been plated on indicator cells of the same age and physiological condition. In an attempt to standardise conditions for plating, late exponential phase cells (OD 0,8) were used to assay irradiated phage samples. Unfortunately, a number of experiments were carried out using cells of different ages before the need to standardise the assay cells was realized. Even when standardised assay cells were used, small variations in the survival curves obtained in different experiments, using the same phage samples, still occurred.

In all later experiments only phages giving clear plaques were used. Three phages (FC2, FC1 and FC5) showing maximum, intermediate and minimum resistance to UV respectively, were selected for further investigation.

9.3.2 HOST CELL REACTIVATION IN *B.FRAGILIS* PHAGES

In order to investigate whether HCR of *B.fragilis* phages occurred, attempts were made to isolate *B.fragilis* HCR$^-$ mutants. A modification of the method of Howard-Flanders & Theriot (1962) was used, using two different phages, FC2 and FC5. Adapting the technique which was designed for *E.coli* for use with the anaerobe *B.fragilis* proved difficult as there were many variables to be considered. However, conditions were obtained which gave only a few isolated colonies on the assay plate. Although *c.* 300 colonies for each of the two phages used were isolated and tested for increased sensitivity to UV radiation, no UV sensitive or HCR$^-$ mutants were obtained.

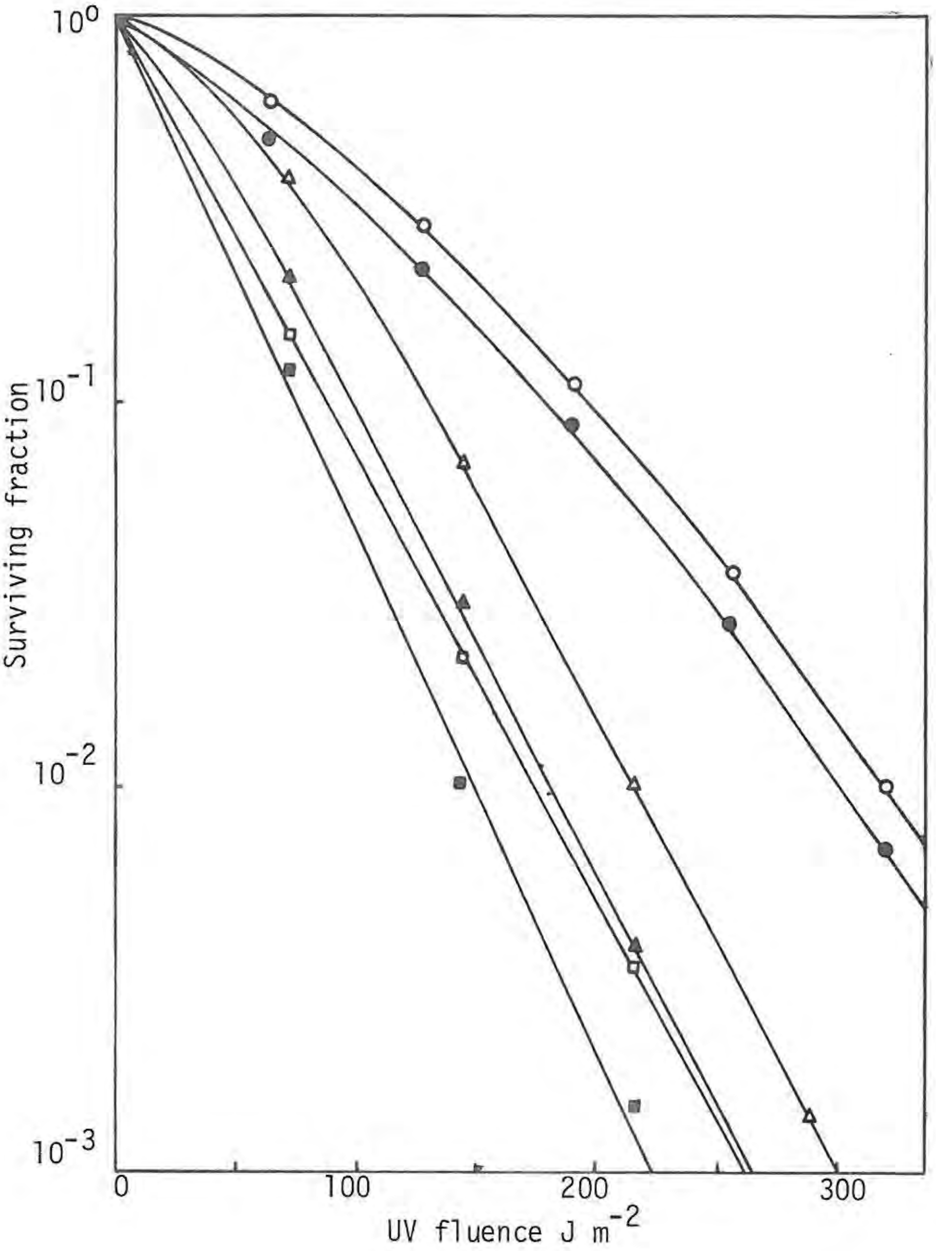


FIG. 9.5: A comparison of the survival of phages FC2 (o●), FC1 (Δ▲) and FC5 (□■) after plating onto BHI plates (open symbols) and BHI plates plus 1 mg ml^{-1} caffeine (closed symbols).

As caffeine is known to inhibit HCR (Metzger, 1964; Sauerbier, 1964), it was added to the BHI agar plates used to assay for infective centres. In irradiated phages plated onto BHI plates with and without caffeine, a small decrease in the surviving fraction of the three phages tested (FC1, FC2 and FC3) was found to occur in the presence of caffeine (Fig. 9.5). The decrease in survival levels obtained, varied, giving a DEF between 1,06 (FC2) and 1,21 (FC1) at 1% survival.

9.3.3 UV REACTIVATION IN *B. FRAGILIS* PHAGES

Low fluences of UV radiation given to the host cell prior to infection by irradiated phages is known to bring about an increase in the survival of certain phages. To investigate whether UVR occurred in some *B.fragilis* phages, *B.fragilis* cells were irradiated with both fixed and increasing fluences of UV irradiation under aerobic and anaerobic conditions, and then infected with irradiated FC1 and FC2 phages. In the first experiment, suspensions of exponential phase indicator cells were irradiated with increasing fluences of UV radiation under both anaerobic and aerobic conditions, and were then infected with suitable dilutions of FC2 phage which had been irradiated with a fluence of 300 J m^{-2}. The irradiated phage assayed on cells irradiated under anaerobic conditions showed a 2,5 fold increase in the surviving fraction of phages in cells irradiated with *c.* 25 J m^{-2} UV irradiation (Fig. 9.6). In cells irradiated with higher fluences a decrease in phage survival occurred. Irradiated phage assayed in cells which had been irradiated under aerobic conditions showed little or no increase in the surviving fraction at lower fluences and at higher fluences the surviving fraction also decreased (Fig. 9.6). In a second

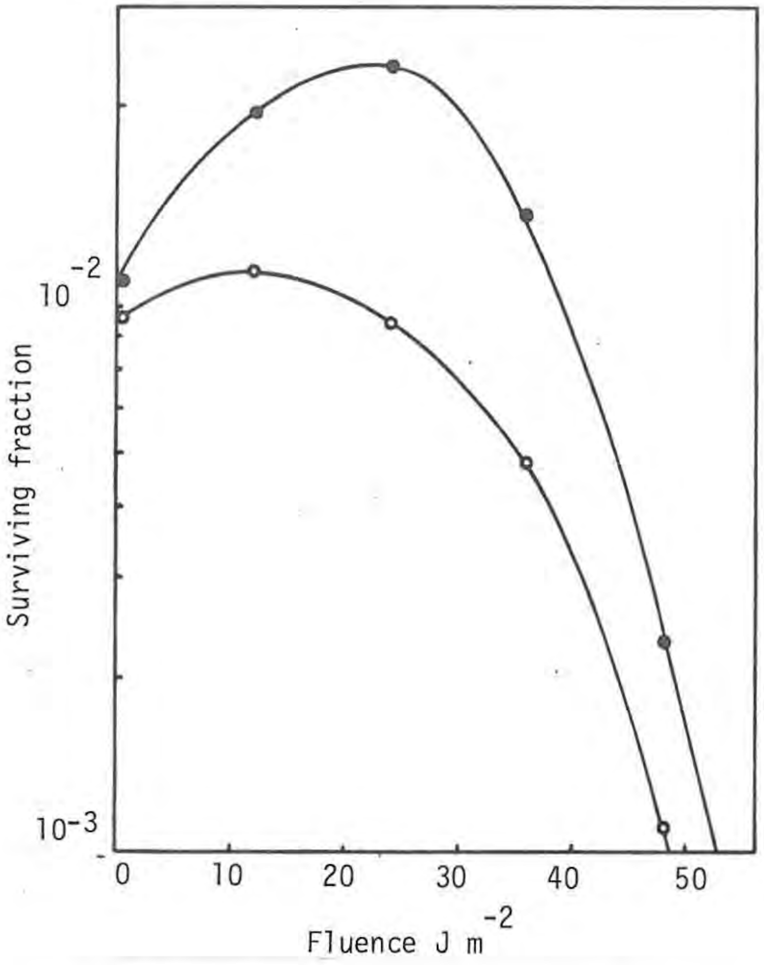


FIG. 9.6: Survival of UV irradiated phage FC2 given a constant fluence of UV radiation (300 J m^{-2}) on UV irradiated *B.fragilis* cells given increasing fluences of UV radiation. Cells were irradiated and assayed under aerobic (o) and anaerobic (●) conditions.

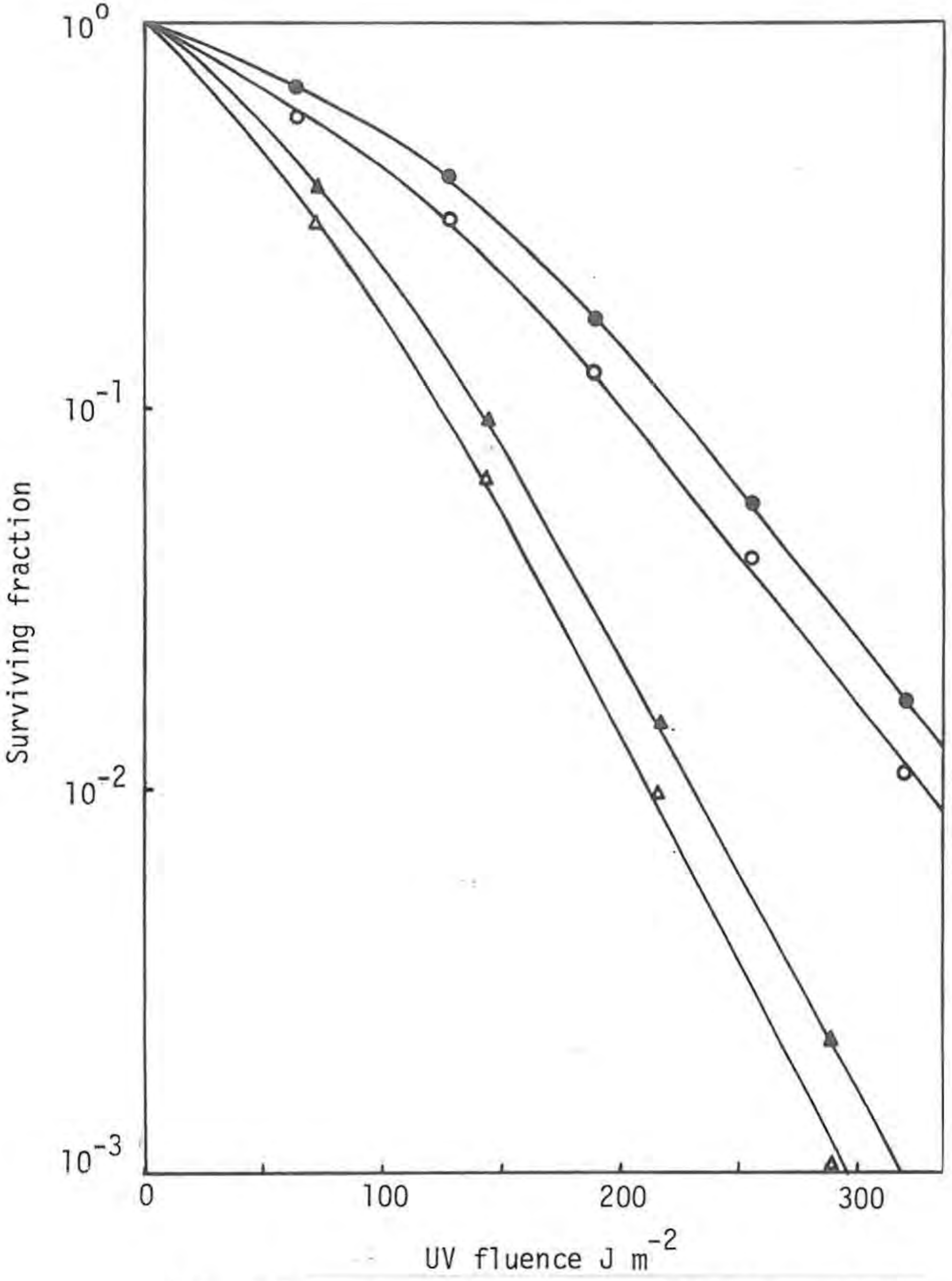


FIG. 9.7: Survival of phages FC1 (△▲) and FC2 (o●) irradiated with increasing fluences of UV. Phages were assayed on unirradiated cells (△o) and on cells irradiated with an UV fluence of 25 J m^{-2} (▲●).

type of experiment the cells used to assay for the surviving fraction of phages were irradiated with a fixed fluence of 25 J m^{-2} and the phages FC1 and FC2 were irradiated with increasing fluences. These phages were then assayed using both irradiated and unirradiated cells and the surviving fraction determined (Fig. 9.7). In both phages, a small increase in the surviving fraction was found in the irradiated phages which had been plated onto the host cells irradiated with low fluences of UV radiation (DRF 0,9 at 1% survival).

9.3.4 MULTIPLICITY REACTIVATION AND MARKER RESCUE IN *B. FRAGILIS* PHAGES.

The occurrence of MR in *B.fragilis* phages was investigated as MR has been used as a test for recombination ability in other phage host cell systems (Huskey, 1969). Irradiated FC2 phages were absorbed onto *B.fragilis* cells to give monocomplexes (m.i.o. *c.* 0,01),and multicomplexes (m.o.i. *c.* 5), under both aerobic and anaerobic conditions. When the m.o.i. is much < 1, the chance of obtaining multiple infected cells is very low and when phages are irradiated with increasing fluences, the rate of inactivation proceeds according to simple exponential kinetics. When bacteria are infected with irradiated phages at a m.o.i. > 1, the ratio of the survivors of multiple infections to those of single infections would be expected to be equal to the m.o.i. (Huskey, 1969). However, in a number of bacteriophages this is not the case, and the ratio of survivors of multiple infections to those of single infections increases with increasing fluence, to values much greater than the multiplicity used. This phenomenon is termed MR, and the most widely accepted explanation of MR is that recombination between two damaged phage genomes in multiple infected cells may lead to an undamaged genome, and eventually to an infective centre.

When FC2 phages were irradiated with increasing fluences of UV radiation and assayed under aerobic conditions, followed by incubation of the plates under anaerobic conditions, the ratio of multicomplex to monocomplex survival remained fairly constant (Fig. 9.8). However, when irradiated FC2 phage was assayed under completely anaerobic conditions, the ratio of multicomplex survival to monocomplex survival increased with increasing UV fluences suggesting that some MR occurred in multiple infected cells under anaerobic conditions.

Marker rescue (cross reactivation) has also been used to demonstrate recombination in bacteriophages. As this type of reactivation depends on the recombination of genetic markers between two phage strains, attempts were made to isolate stable phage mutants, both after treatment with 5-bromouracil and in UV irradiated phages assayed under anaerobic conditions. Three main types of plaque variants were isolated, those showing marked differences in size (either very small or very large plaques), those showing differences in the edge of the plaque (either fuzzy or sharp, clear edged plaques), and thirdly, those phages which possessed either turbid halo's or shadowy turbidity within the plaque itself. Although over 200 plaques showing apparent differences in morphology were isolated, none of them were stable mutants. No attempts to obtain other types of phage mutants (such as host range sensitivity mutants) were made. As no mutants were isolated it was not possible to attempt marker rescue with a *B. fragilis* phage.

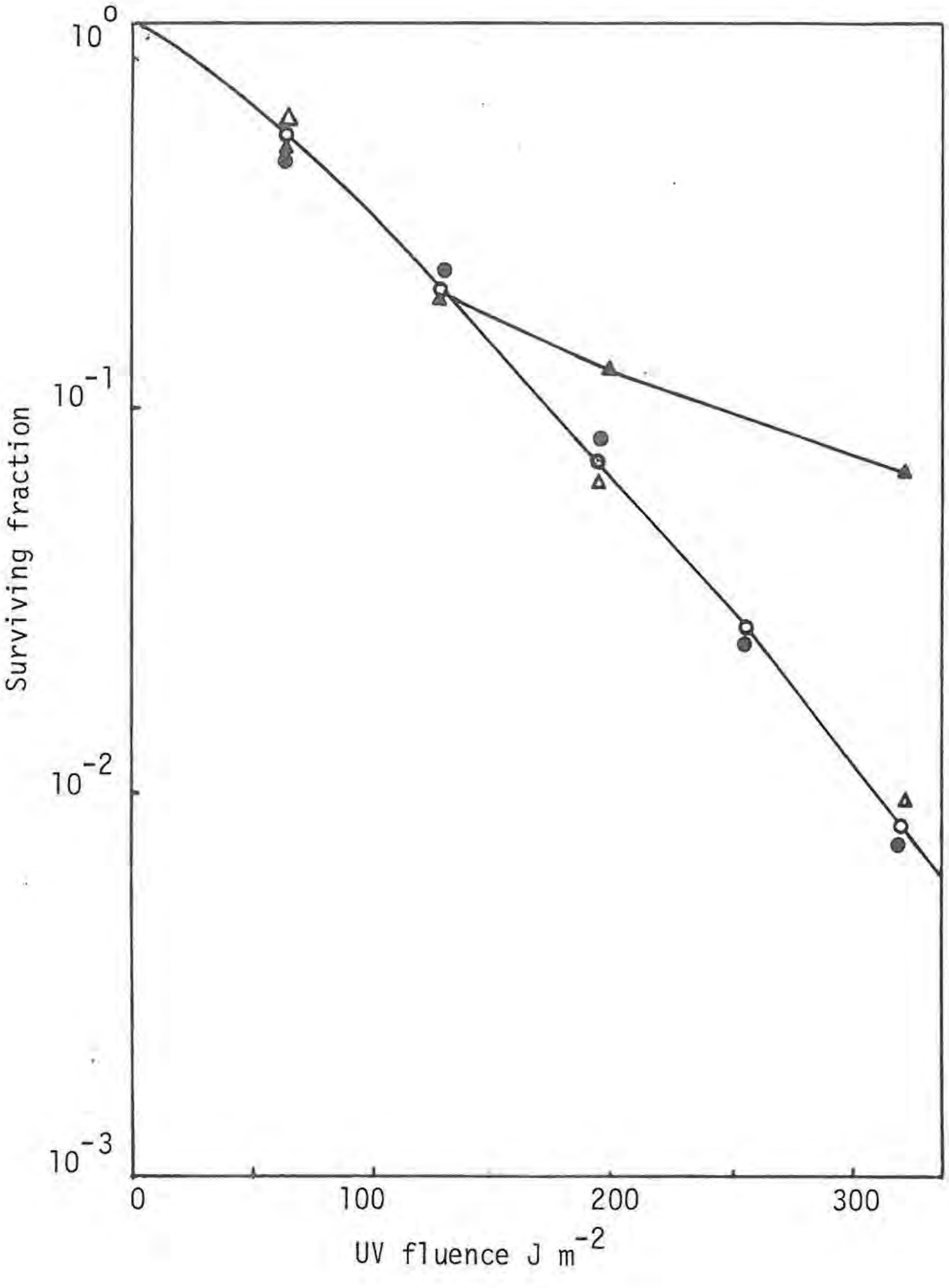


FIG. 9.8: Inactivation of UV irradiated FC2 phage adsorbed onto *B.fragilis* cells at m.o.i. of *c*.0,01 (monocomplexes) (o●) and a m.o.i. of *c*.5 (multicomplexes) (Δ▲). Phage was assayed under aerobic (oΔ) and under anaerobic (●▲) conditions.

9.4 DISCUSSION

Bacteriophages damaged by UV irradiation may be repaired in the host cell by a variety of repair processes. Phage reactivation processes which are dependent on all 3 of the major host cell repair systems are known, as well as some which require additional enzymes coded for by certain phages. Thus, phages have proved to be extremely useful tools with which to study recovery and repair systems in the host cell. Before attempting to utilize *B.fragilis* phages as a means of investigating recovery and repair processes in *B.fragilis* cells, it was first necessary to determine the sensitivity of the individual phages to UV radiation.

In 7 of the *B.fragilis* phages the log of the surviving fraction was found to decrease linearly with the fluence of radiation. Thus, the rate of inactivation proceeded according to simple exponential kinetics. In the other 5 phages slight shoulders were present, however, in all cases the extrapolation number was <1. The UV sensitivity of these phages varied over a 2,25-fold range and all were markedly more resistant to UV radiation than the T phages of *E.coli*. The presence of a small shoulder in some of the phages does not appear to have much significance. Although most phages show exponential inactivation kinetics, the survival curves of many phages do show shoulders. The most common explanation for this is that it takes more than one hit to inactivate the phage, resulting in multi-hit kinetics.

Phages are known to vary greatly in their sensitivity to UV radiation, and the rate at which they are inactivated depends largely on the intrinsic sensitivity of the phage DNA to UV radiation (Stent, 1963).

In addition, the degree to which the damaged DNA is reactivated in the host cell also markedly affects the number of viable phages in the surviving fraction (Swenson, 1976).

In an attempt to determine whether HCR occurred in *B.fragilis*, host cells infected with irradiated phages were plated out onto BHI agar containing caffeine, as caffeine is known to inhibit HCR in *E.coli* (Metzger, 1964; Sauerbier, 1964). A small decrease in the number of infective centres occurred when infected *B.fragilis* cells were plated onto media containing caffeine. Although the effect was slight, it was consistently obtained under all conditions. The reason for the small decrease in survival is not known but it could be due to inhibition of a HCR process which is only responsible for a very small degree of reactivation. Another possibility is that no HCR occurs in the phages tested and that the caffeine interfered with some other function in the host cell or phages leading to an increase in phage inactivation. In view of the striking effect of caffeine observed in the experiments on cell survival and LHR in *B.fragilis*, it would appear that host cell excision repair processes had little or no effect in bringing about HCR in the phages tested. It is not known if any phage coded repair process is involved in the relatively high resistance to UV radiation in *B.fragilis* phages. Further elucidation of the role of excision repair processes in phage reactivation in *B.fragilis* would be greatly facilitated by the isolation of repair deficient host cell mutants and radiation sensitive phages. However, as no HCR or excision repair mutants were isolated, it was not possible to investigate phage survival in repair deficient cells.

A number of experiments were performed to determine whether UVR occurred in *B.fragilis*. In two of the phages tested, a small increase in survival of irradiated phages which were assayed in host cells subjected to low fluences of UV radiation, was found to occur under anaerobic conditions, but not under aerobic conditions. In irradiated FC2 phage a 2,5-fold increase in survival occurred at a fluence of 25 Jm^{-2} and the survival curves of the 2 phages showed a DRF of slightly less than 0,9. UVR is dependent on functional *rec* A and *lex* gene products and has been found to occur in a number of *E.coli* phages. The absence of UVR under anaerobic conditions indicates that similar functions in *B.fragilis* may be inhibited in the presence of oxygen.

As MR in *E.coli* is known to require a functional recombination system, experiments were carried out in *B.fragilis* to try and ascertain whether MR of irradiated phages could occur in this strain under anaerobic or aerobic conditions. Difficulties were experienced in carrying out the complex procedures, necessary in MR experiments, in a glove box under anaerobic conditions. However, the results obtained with both the FC1 and FC2 phages indicate that some MR did occur in *B.fragilis* under strictly anaerobic conditions but not under aerobic conditions. Huskey (1969) showed that MR in λ is dependent on the recombination function of either the host bacterial cell or the phage. Either one is sufficient to bring about MR but in the absence of both systems MR is inhibited. His work indicates that MR can be used as a method for detecting the presence of recombination systems in bacteria or bacteriophages without the requirement of having genetic markers. If the MR phenomenon observed in *B.fragilis* is also due to a recombination process, these results suggest that such a recombination function may only occur under anaerobic conditions in this organism. The lack of success in obtaining any stable phage mutants prevented marker rescue experiments being done. These experiments might have provided further information on genetic recombination processes in *B.fragilis*.

Although direct comparisons of repair processes occurring in *E.coli* and *B.fragilis* may not be valid or relevant, it is interesting that both UVR and MR which require a functional *rec* A system in *E.coli* only occur under strictly anaerobic conditions in *B.fragilis*. It is also of interest to note that even under strictly anaerobic conditions, the level of phage reactivation obtained in *B.fragilis* cells appeared to be less than the reactivation obtained in *E.coli* systems. The results obtained from the UVR and MR experiments coupled with the evidence obtained from the LHR experiments, suggest that processes which require a *rec* A type of function, can probably only occur in *B.fragilis* when cells have been maintained under strictly anaerobic conditions. The significance of these findings are discussed in Chapters 10 and 11.

CHAPTER X

REPAIR OF UV DAMAGE IN *B. FRAGILIS* AND ITS RELATIONSHIP TO MUTAGENESIS.

10.1 INTRODUCTION

One of the most important discoveries in the field of photobiology has been that cells can recover from radiation induced damage. The recovery from such damage can be influenced both by pre- and post-irradiation conditions to which cells are subjected, and to a large extent the differences in the amount of recovery which occurs is dependent on the efficiency of the repair processes. The induction of mutations has also been found to be dependent on the nature of the repair processes involved.

Three main modes of DNA repair are known. In the first type of repair, the lesion in the DNA molecule is restored to its functional state *in situ*. This may be accomplished by an enzymatic mechanism as in the case of photoreactivation, where the *in situ* and enzymatic splitting of cyclobutyl pyrimidine dimers is mediated by exposure of the enzyme-dimer complex to visible light. Repair of some types of DNA damage may also occur by spontanerous 'decay' of the damage to an innocuous form, as in the case of the dehydration of pyrimidine photohydrates or the recombination of radicals to yeild a restored molecule (Smith, 1978b).

In the second type of repair process, the damaged section of the DNA strand is excised and replaced with undamaged nucleotides. This type of repair is known as excision repair, and it was first identified as the repair process responsible for the removal of UV induced cytobutyl pyrimidine dimers in the absence of light (dark repair) (Boyce & Howard-Flanders, 1964). It has since been shown that excision repair mechanisms are responsible for the repair of a variety of radiation and chemically induced lesions in DNA.

In the third type of repair process, the damage itself is not repaired but instead, it is bypassed during replication, leaving gaps in the daughter strand DNA. The missing genetic information is subsequently supplied by recombination with the original parent strand. The parent strand is then repaired by repair synthesis. This type of repair has been called post-replication repair since it can occur only after DNA synthesis has taken place.

Much of the knowledge and insight into the complexities of DNA repair have come from the study of bacterial systems. The isolation and characterization of the diverse systems of repair has been made possible by the availability of numerous radiation sensitive mutants, and the techniques which permit the construction of the multiple repair-deficient mutants (Clark & Ganesan, 1975; Bachmann *et al.*, 1976). The use of such mutants has lead to the characterization of DNA repair processes at the molecular level. Studies using multiple mutants (especially those constructed in *E.coli*) have shown that both excision repair and post-replication repair processes are composed of multiple biochemical pathways. Some of these pathways appear to be error free, while others appear to be error prone. UV induced mutagenesis results from mistakes made during the repair of damaged DNA by these inducible error prone pathways. In general, post-replication repair appears to be more error prone then excision repair (Smith, 1978b).

10.1.2 PHOTOREACTIVATION

The photoreactivation phenomenon was discovered by Kelner (1949). He found that the survival of UV-irradiated *E.coli* B could be greatly enhanced if the cells were exposed to an intense blue light. Rupert *et al.* (1958) obtained photoreactivation in an *in vitro* system, and demonstrated the existence of the photoreactivating enzyme and established its basic properties and made of action (Rupert, 1962a, 1964). The extensive literature which has subsequently appeared dealing with photoreactivation has been reviewed by Rupert (1964),Setlow, (1966, 1967b), Cook (1970), Harm *et al.* (1971), Rupert (1975) & Friedberg *et al.*(1977).

Photoreactivation is carried out by a single enzyme which in most cases has been found to consist of a single polypeptide chain with a molecular weight of 35 000 - 40 000 d (Sutherland, 1978). The photoreactivating enzyme combines specifically with cyclobutyl pyrimidine dimers in UV-irradiated DNA to form a substrate enzyme complex. Binding to the substrate resembles other specific proteins which bind to nucleic acid, and binding to single stranded DNA can occur if it is of sufficient length (Harm, 1966). The complex can form in the absence of light but activation of the complex to convert the dimers back to monomeric pyrimidines, must be activated by light of wavelength 320 to 410 nm). This is illustrated schematically in Fig. 10.1. Although enzyme-bound sensitizers with an absorbtion around 360 nm have been postulated, none have been found. Instead, it appears that the absorption spectrum of the complex is generated during binding and is dependent on the structure of the nucleic acid enzyme complex (Harm, 1975). Under certain experimental conditions as much as 80% of the lethal damage induced in bacteria by low fluences of UV radiation can be photoreactivated (Setlow, 1966). In addition to many species of prokaryotes, photoreactivation has also been found to occur in numerous eukaryote groups (Cook, 1970; Rupert, 1975).

10.1.2 EXCISION REPAIR

Excision repair was first discovered in *E.coli* B by Setlow & Carrier (1964) and in the K-12 strain by Boyce & Howard-Flanders (1964). According to present models the first step in the excision repair process is the recognition of the damaged nucleotides and the introduction of a break in the DNA chain near the lesion (incision step). Resynthesis is then initiated by the action of DNA polymerase (repair replication) using the opposite strand of the DNA as the template. During this step the lesion is cut out completely and replaced with undamaged nucleotides and the single-strand gap is finally closed enzymatically by polynucleotide ligase to yield repair DNA. Although this has been shown to be the general mode of repair for the major excision repair pathway, it is not the only pathway by which excision repair can take place.

The mutants *uvr* A, *uvr* B and *uvr* C were among the first radiation sensitive mutants to be mapped in *E.coli*. Although the genes are widely dispersed on the chromosome (Howard-Flanders *et al.*, 1966) the three mutants all have similar phenotypes in that they fail to excise dimers from irradiated DNA. The *uvr* A and *uvr* B genes have been shown to code for the enzyme responsible for the recognition of pyrimidine dimers and for making the incision break (endonucleic step on the 5' side of the dimer). This enzyme, known as

FIG. 10.1: A GENERAL MODEL FOR PHOTOREACTIVATION

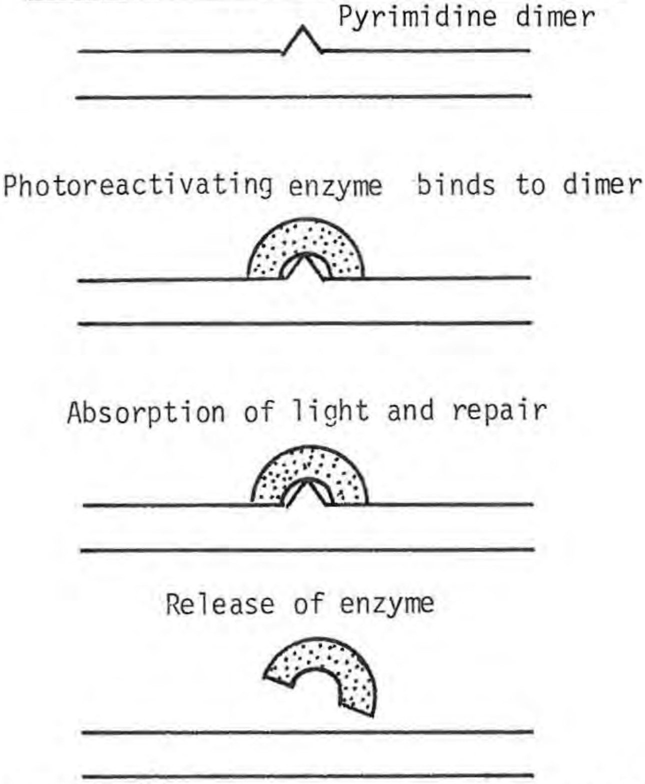
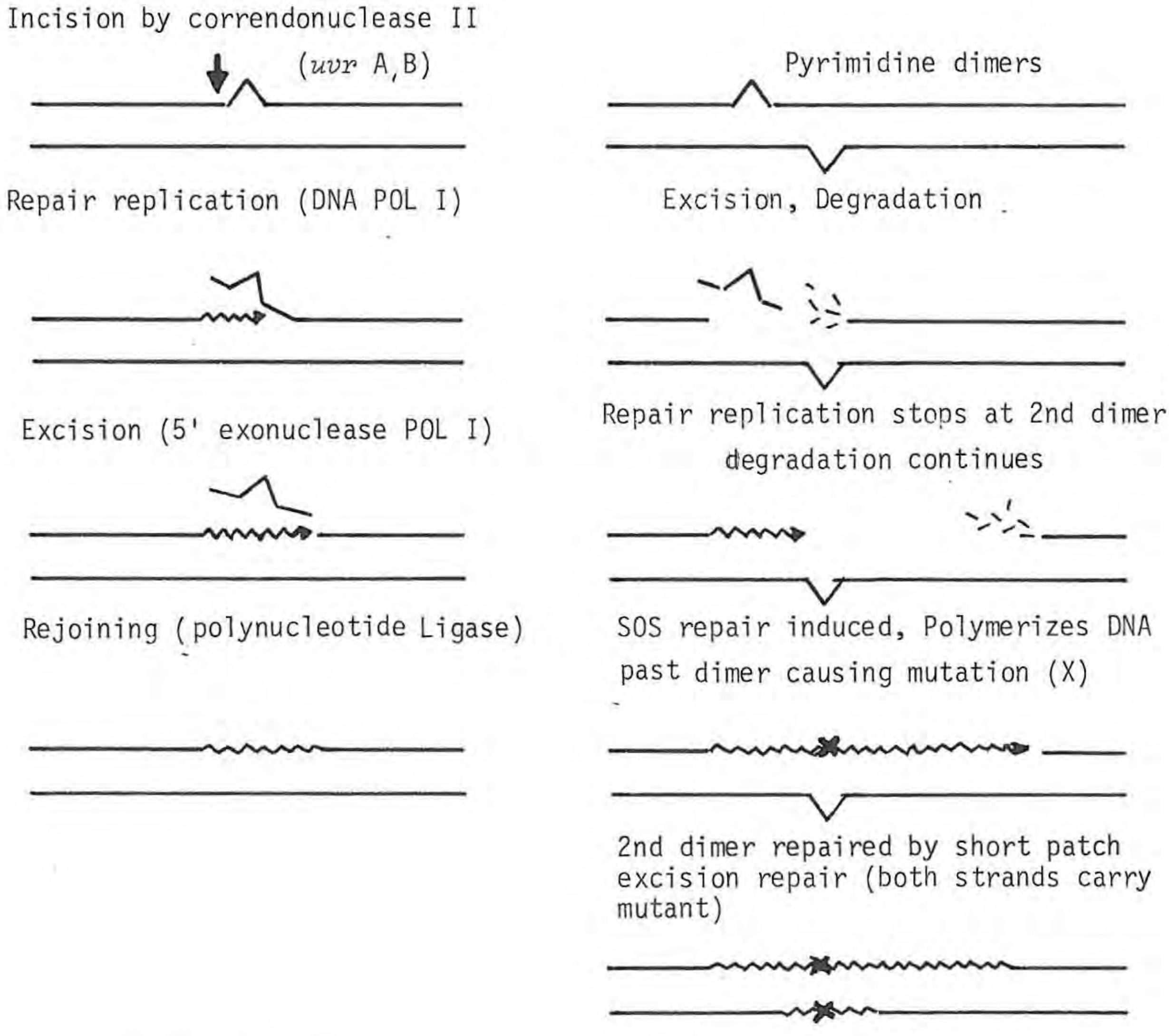


FIG. 10.2: GENERAL MODEL FOR THE TWO MAJOR PATHWAYS OF EXCISION REPAIR



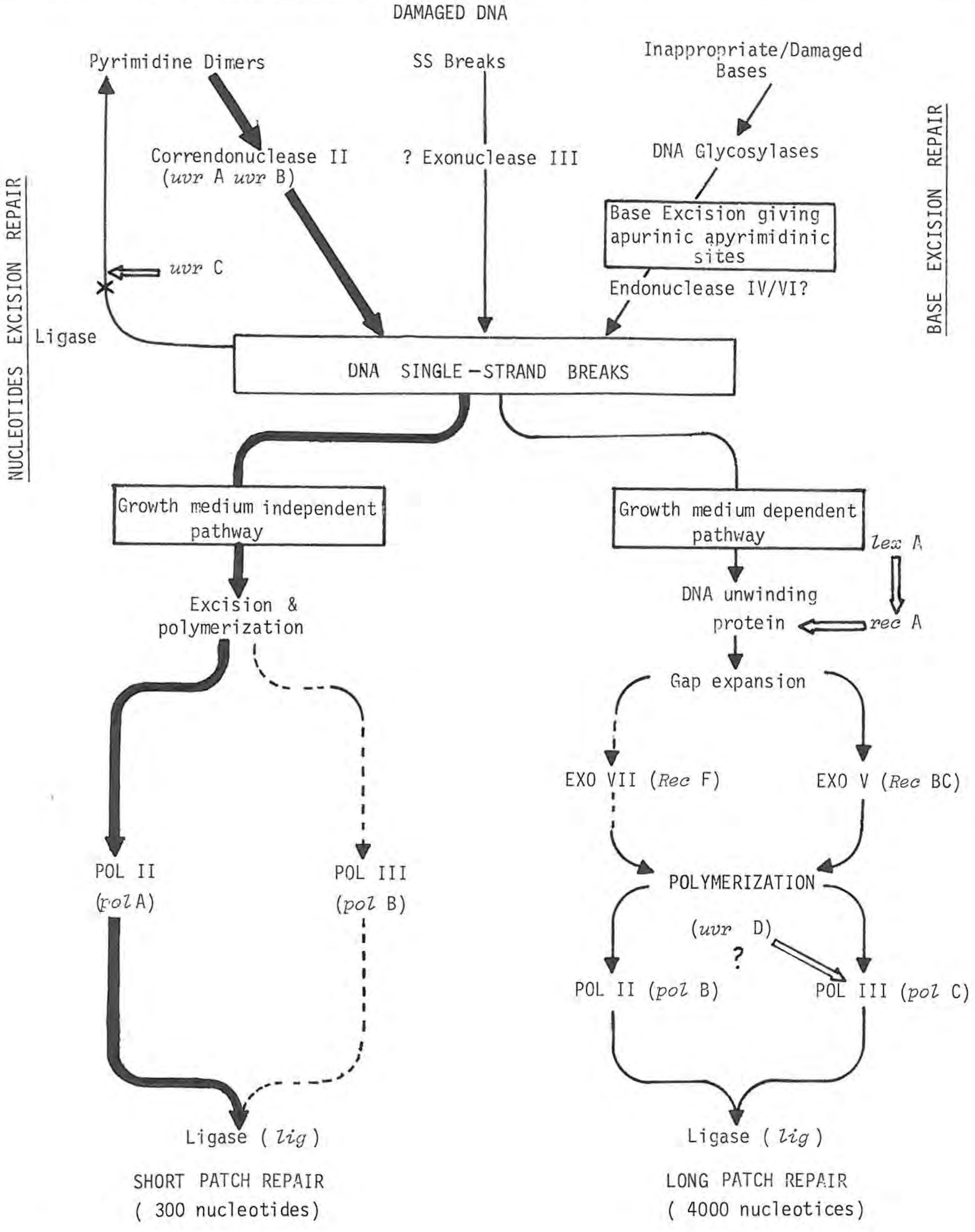
(A) SHORT PATCH PATHWAY (B) LONG PATCH PATHWAY

(after Smith, 1978; Witkins, 1976).

correndonuclease II was identified by Braun & Grossman (1974) and has been isolated and partially characterized (Braun *et al.*, 1975). In strains carrying the *uvr* C mutation, most of the incisions produced by correndonuclease II are apparently resealed by polynucleotide ligase (Seeberg & Rupp, 1975) thus preventing repair synthesis taking place. The *uvr* C gene product appears to interact with the ligase system to prevent abortive sealing of the endonuclease breaks (Fig. 10.3). The incision step is then followed by repair synthesis. This excision repair process can occur via two major pathways, which can be distinguished both on the bases of the gene products involved and on the physiological requirements (Figs. 10.2 & 10.3).

The one pathway appears to produce short patches of repair synthesis (less than 30 nucleotides) hence it has been termed the short patch pathway (Cooper & Hanawalt, 1972). In this pathway, repair can occur in buffer, and thus it is independent of nutrients and is not inhibited by the presence of chloramphenicol. This pathway requires DNA polymerase I (*pol* A^+) and it has been shown to be capable of both the excision and the polymerization steps needed for repair synthesis (Kelly *et al.*, 1969) (Fig.10.3). However, in *pol* A^- strains, DNA polymerase III (*pol* C) and under certain experimental conditions DNA polymerase II (*pol* B) can partially substitute for polymerase I, being capable of repairing about 25% of the lesions (Youngs *et al.*, 1974). However, as *pol* B mutants are not radiation sensitive it is probable that repair synthesis can only be carried out by either DNA polymerase I or III *in vivo*. The short patch pathway appears to be both constitutive and error free, and is the mode of repair responsible for most excision repair

FIG. 10.3: THE GENETIC AND PHYSIOLOGICAL CONTROL OF DIFFERENT PATHWAYS OF EXCISION REPAIR



(Youngs *et al.*, 1974) including LHR and HCR. The finding of Monk *et al.* (1971) that *pol* A *uvr* A strains are only slightly more sensitive to UV radiation than *uvr* A strains provides strong evidence that most excision repair is accomplished via the *pol* A^+ dependent pathway.

A second excision repair pathway which produces long patches of repair synthesis (*c.* 1 500 nucleotides) is also known. This pathway requires a complete growth medium and is irreversibly inhibited by post-irradiation treatment with chloramphencol (Youngs *et al.*, 1974). This pathway has been shown to require functional *rec* A, *rec* B, *rec* C, *lex* A, *pol* C and *leg* genes (Youngs & Smith, 1973 a; Youngs *et al.*, 1974; Youngs & Smith, 1977). In addition *rec* F, *pol* B and *uvr* D gene products also appear to be involved in alternate branches of this pathway (Smith *et al.*, 1978). The actual steps involved in long-patch repair are not known. In the light of the most recent evidence it is possible that the *rec* A gene product is responsible for unwinding the DNA (Cunningham *et al.*, 1979) and the *lex* A gene product is involved in the regulation of the *rec* A gene (Bailone *et al.*, 1979). The *rec* B, *rec* C gene product is known to be exonuclease V and the *rec* F gene product is known to be exonuclease VIII (Eisenstark, 1977). Gap expansion in long patch repair appears to be brought about predominantly by the *rec* B, *rec* C gene product but in the absence of exonuclease V a second minor pathway utilizing the *rec* F gene product appears to function (Witkin, 1976). Both DNA polymerase II and III (*pol* B and *pol* C) appear to be able to function in the polymerization step in long patch repair and at the moment their separate roles are not understood (Smith *et al.*, 1978). In addition there appears to be both *uvr* D dependent and independent pathways involved in this repair process (Smith *et al.*, 1978) and present evidence suggests that the *uvr* D gene product may serve to regulate either DNA polymerase II or III (Kushner *et al.*, 1978).

In addition to the excision of pyrimidine dimers, excision repair processes have also been found to be involved in the repair of other types of DNA lesions. Single strand breaks produced by X-raysand other agents have also been shown to be repaired by the same general pathways just described (both short patch and long patch) (Smith, 1978b). Although correndonuclease II (*uvr* A & B) is not involved in these repair processes other endonucleases such as endonuclease III may play a role (Smith, 1978b). Double stranded breaks in DNA do not appear to be repairable in *E.coli* and are lethal to the cell (Bonura & Smith, 1975). The excision repair system which is responsible for the excision of pyrimidine dimers is known as nucleotide excision repair, as the whole of the damaged region is excised. A second type of excision repair process has been characterized which has been termed base excision repair (Friedberg *et al.*, 1978). Base excision repair is a specific DNA repair mode which functions to remove incorrect or altered bases from the DNA. The inappropriate or damaged bases are excised as free bases by enzymes termed DNA glycosylases which hydrolyse the bond linking the base to the sugar phosphate backbone leaving apurinic-apyrimidinic gaps in the DNA (Friedberg *et al.*, 1978). *E.coli* has been shown to contain several DNA glycosydases, each of which is highly specific for a particular type of lesion, and they appear to function to edit mutagenic deaminated residues in the DNA (Lindahl *et al.*, 1978). The action of this editing process appears to preclude the use of uracil as a normal DNA constituent, as well as excising other inappropriate bases from the DNA. The sites of base loss are then thought to be attacked by specific apurinic-apyrimidinic endonucleases (e.g. endonuclease IV & VI) which create free ends for subsequent exonucleolytic excision of the sugar-phosphate residue. Excision and resynthesis is thought to occur via the same repair process involved in nucleotide excision repair (Smith, 1978b).

10.1.3 POST-REPLICATION REPAIR

The third major mode of repair of UV damage is post-replication repair which was discovered by Rupp & Howard-Flanders (1968). They observed that the DNA synthesised immediately following UV irradiation in *E.coli* K-12 cells, contained discontinuities when assayed in alkaline sucrose gradients. In excision deficient cells, the mean length of newly synthesized daughter strand DNA was found to approximate the average distance between pyrimidine dimers in the parental strand. After a period of growth, these discontinuities were found to disappear and the DNA was again found to approximate the molecular size of that from unirradiated control cells. They suggested that a post-replication repair process was involved in which DNA replication could proceed past the lesions in the parental strand leaving gaps in the daughter strand DNA opposite the dimers in the parental strand. These gaps would then be filled by a recombination process using an intact strand of DNA obtained from the parental strand. The gaps left in the parental strand would then be filled by repair synthesis This repair process is shown schematically in Fig. 10.4. The size of the daughter strand gaps left in the newly synthesized DNA appear to be about 1 000 nucleotides long (Iyer & Rupp, 1971) and evidence has been obtained that parental and daughter strand DNA become covalently attached during post-replication repair (Rupp *et al.*, 1971). Observations involving excision-repair deficient cells, whereby approximately half of the sites in the UV-irradiated DNA that are sensitive to the action of the T4 UV endonuclease (pyrimidine dimers) are transferred from the parental strand to daughter strand during post-replication repair, strongly suggests that recombination is involved (Ganesan, 1974). It appears that in excision repair-deficient cells it takes several rounds of replication and post-replication repair before pyrimidine dimers are 'diluted out' sufficiently so that a viable strand of DNA is obtained (Bridges & Munson, 1968; Ganesan & Smith, 1971). A number of genes have been shown to be involved in the post-replication repair process. These include *rec* A, *rec* B, *rec* C, *rec* F, *lex*, *uvr* D, *pol* A and *pol* C (Smith, 1978b). Little is known about the actual role of these gene products and the mechanisms involved, but there is evidence to suggest that a number of different pathways exist in post-replication repair processes. The enzymes involved in these repair processes are probably common to those used in some steps in recombination, and are also similar to those which opperate in growth medium dependent excision repair (Swenson, 1976). It is possible that *rec* A and *lex* are involved in DNA unwinding processes (Cunningham *et al.*, 1979) while *rec* B, *rec* C and *rec* F gene products are exonucleases involved in gap expansion (Eisenstark, 1977) and *pol* A, *pol* C and

POST-REPLICATION REPAIR

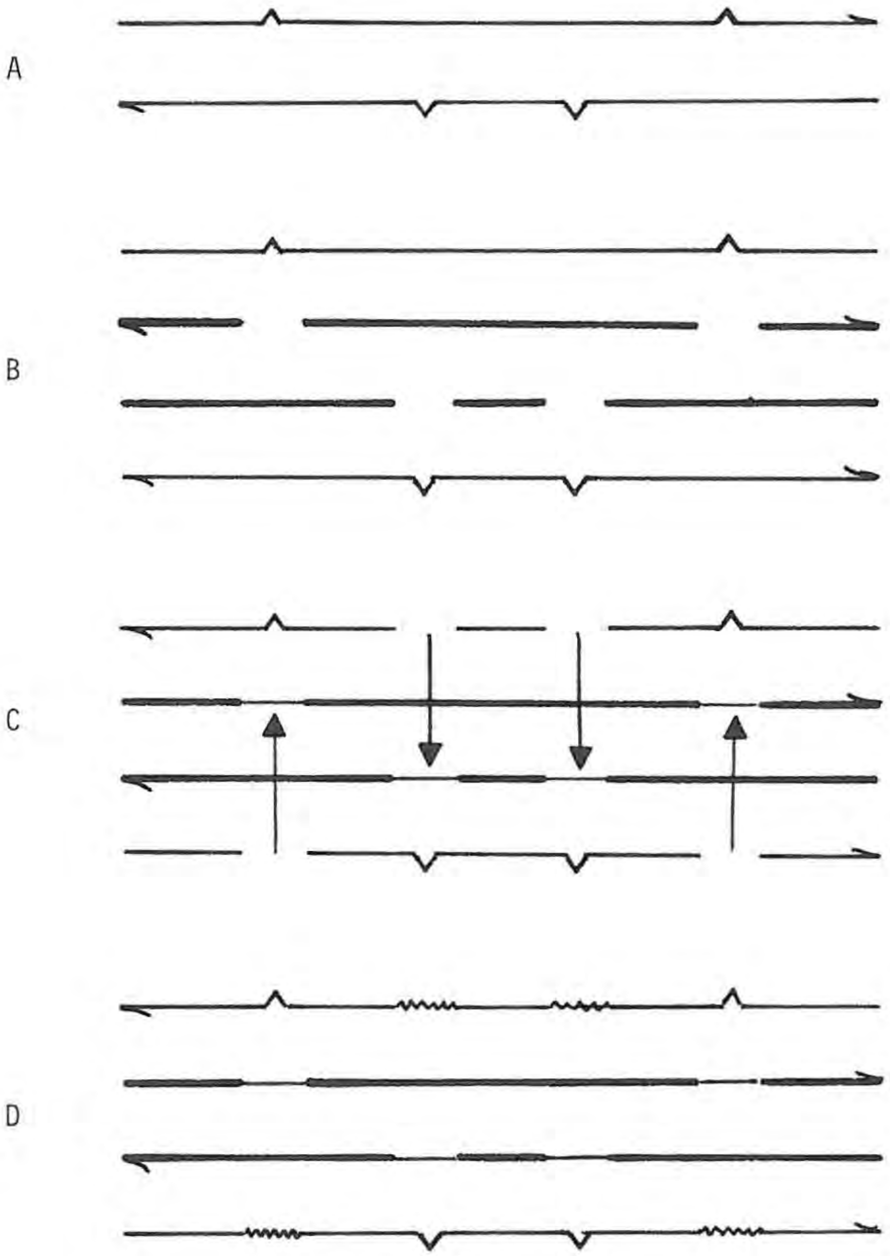


FIG. 10.4: A GENERAL MODEL FOR POST-REPLICATION REPAIR OF DNA DAMAGED BY UV RADIATION

(A) Pyrimidine dimers produced in the two strands of DNA.

(B) DNA synthesis proceeds past the lesions in the parental strands leaving gaps in the daughter strands.

(C) Filling of the gaps in the daughter strands with material from the parental strands by a recombination process.

(D) Repair of the gaps in the parental strands by repair replication.

(after Smith, 1978)

uvr D gene products are involved with the repair synthesis steps (Bridges, 1978; Kushner *et al.*, 1978). The filling of the gaps in newly synthesized daughter strands of DNA is inhibited in *rec* A mutants (Smith & Meun, 1970; Sedgwick, 1975a), but *rec* B, *uvr* D and *lex* A mutants only cause partial inhibition (Smith, 1978b). Chloramphenicol which partially inhibits post-replication repair is not effective in the three latter mutants (Smith, 1978b). Efficient post-replication repair can occur in either *pol* A or *pol* C strains but is absent in *pol* A, *pol* C strains (Sedgwick & Bridges, 1974; Tait *et al.*, 1974). It is not known which of the two polymerases are used preferentially on the wild type cell. There is evidence that *pol* C is required for error prone repair to occur (Bridges, 1978). Polynucleotide ligase is also required for the completion of post-replication repair (Youngs & Smith, 1977).

10.1.4 RELATIONSHIP OF REPAIR TO SURVIVAL AND MUTAGENESIS

In cells which have sustained potentially lethal damage to their DNA the processes of repair, survival and mutagenesis are inseperable. A surviving cell may undergo a mutation, either because an unrepaired DNA lesion generates a replication error or because an error prone repair system has changed the base sequence in the course of restoring a viable DNA structure (Witkin & Kirschmeier, 1978). However, there is still much controversy as to which pathways are involved in error prone repair and which are error free. Regretably studies with multiple repair deficient mutants frequently do not permit an easy answer to the question (Smith, 1978b).

Mutants which lack *rec* A and *lex* function do not undergo mutagenesis, on the other hand, mutants which lack excision repair (uvr^-) are more mutable than wild type cells. Taken together, this indicates that nutrient independent (short patch) excision repair is essentially error free, and that the error prone pathway is dependent on *rec* A and *lex* function. In view of the error free nature of the short patch pathway *pol* A$^-$ cells would be expected to show increased mutation rates. However, experiments involving *pol* A mutants have given conflicting results and have been difficult to interpret (Smith, 1978b).

A hypothesis first proposed by Radman (1974,1975) suggested that *E.coli* possesses an inducible DNA repair system (SOS repair) which is also responsible for induced mutagenesis. Some characteristics of SOS repair are, (1) it is induced or activated following damage to DNA; (2) it requires *de novo* protein synthesis; (3) it requires several genetic functions of which *rec* A and *lex* have been the most intensively studied; (4) the physiological and genetic requirements for the expression of SOS repair are very similar to those necessary for the induction of a number of other apparently inducible systems in *E.coli*. This process is shown schematically in the model shown in Fig. 10.5. These include, UV inducibility of prophage, the induction of mutagenic UVR, delayed cell division (resulting in filamentous growth in *lon*$^-$ mutants), aberant reinitiation of DNA replication at the chromosome origin after arrest, respiration shut off and the synthesis of the *rec* A gene product (Witkin, 1976). All of these phenotypic effects are absent in *rec* A$^-$ and/or *lex*$^-$ cells (Table 10.1).

The *rec* A gene product (protein X) appears to be able to function in a number of ways. It is able to bind to single stranded DNA to prevent

TABLE 10.1: PLEIOTROPHIC EFFECTS OF *REC* A AND *LEX* A MUTATIONS IN *E.COLI*.

PHENOTYPE	Wild Type $recA^+ lexA^+$	*rec* A$^-$	*lex* A$^-$
Recombination ability	+	-	+
UV sensitivity	+	+++	++
Xray sensitivity	+	+++	+++
DNA degradation	+	+++	+++
Thermal inducibility of SOS in *tif* -1 mutants	+	-	-
Synthesis of Protein X	+	-	-
Long patch excision repair pathway	+	-	-
Chloramphenicol sensitivity postreplication repair	+	-	-
UV mutagenesis	+	-	-
UV inducibility of λ prophage	+	-	-
Mutagenic UVR	+	-	-
Delayed cell division (filamentous growth when extreme	+ lon^+ +++ lon^-	- -	- -
Aberrant reinitiation of DNA replication	+	-	?
at chromosome origin after arrest	+	-	-
Respiration shut off	+	-	-

KEY:
- \+ Normal effect
- \- Effect absent
- +++ Enhanced effect

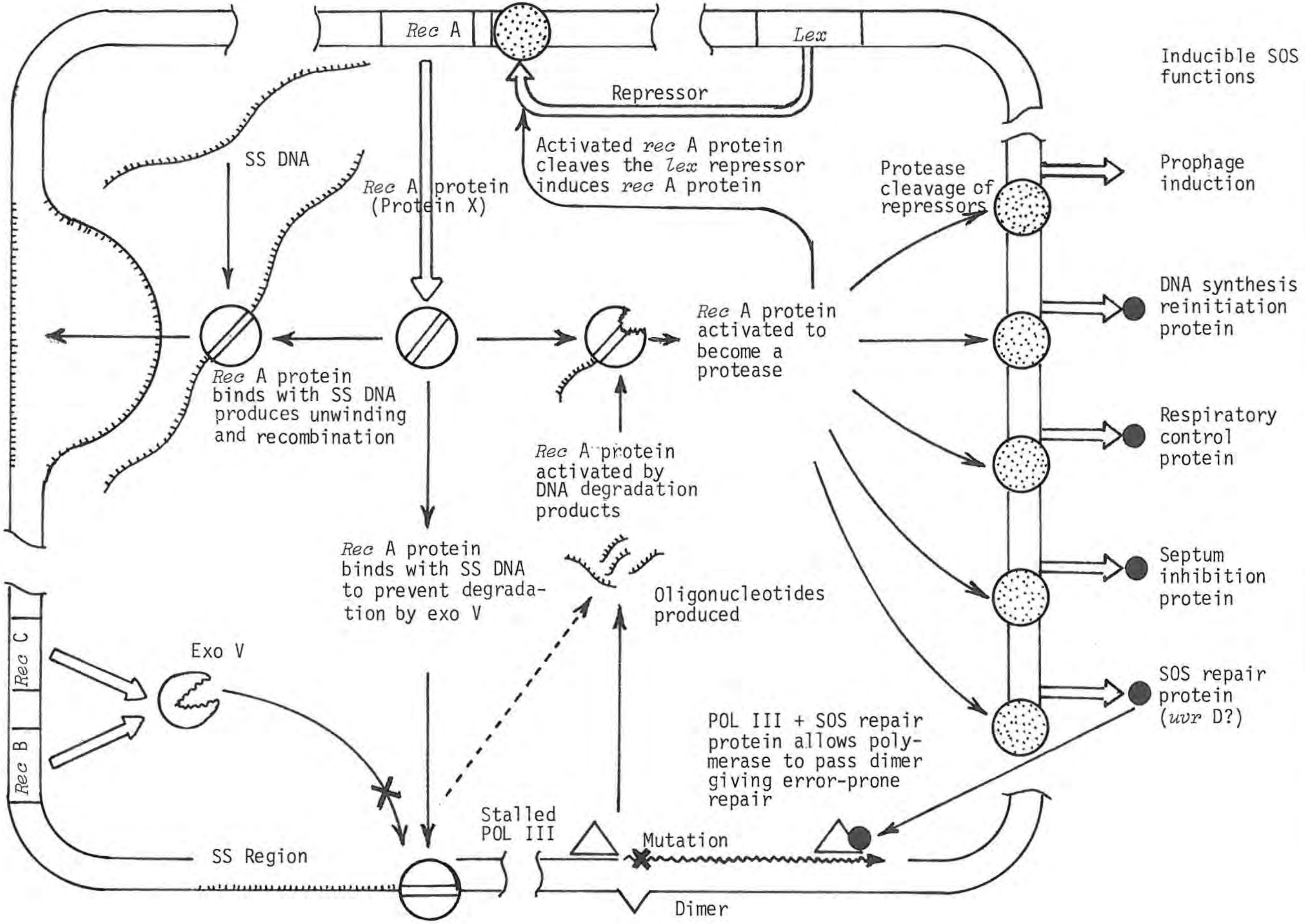


FIG. 10.5: MODEL TO ACCOUNT FOR THE REGULATION OF SOS FUNCTION.

DNA degradation by the *rec* BC coded exonuclease V (Eisenstark, 1977). In addition single-stranded DNA (whether homologous or not) has been shown to stimulate purified *E.coli rec* A protein to unwind duplex DNA (Cunningham *et al.*, 1979). This helps to explain how *rec* A promotes a search for homology in genetic recombination. As oligodeoxynucleotides also stimulate unwinding, a common mechanism may relate the function of *rec* A protein in recombination to other (SOS) functions induced by oligonucleotides (Fig. 10.5). The *rec* A protein has also been shown to inactivate the phage λ repressor *in vitro* (Roberts *et al.*, 1978) and *in vivo* (Bailone *et al.*, 1979). The occurrence of proteolytic cleavage of the λ repressor *in vitro* and *in vivo* (Roberts & Roberts, 1975; Roberts *et al.*, 1977) suggests that proteolytic cleavage may be the mechanism whereby the *rec* A protein inactivates the prophage repressor and other repressors,which lead to the various SOS functions in *E.coli*. Meyn *et al.*(1978) have shown that several protease inhibitors of different chemical structures inhibit SOS-dependent inactivation of λ repressor. In addition, high intracellular concentrations of λ repressor inhibit the expression of error prone DNA repair. The *rec* A protein can apparently only carry out proteolytic cleavage once it has been activated by an effector, possibly an oligodeoxynucleotide or dMNP (Emmerson & West, 1978) and both the unwinding function and the cleavage function require ATP (Cunningham *et al.*, 1979). Accordingly oligonucleotides or single strands may trigger a conformational change of *rec* A protein that can both unwind DNA and alter the interaction of *rec* A protein with certain other proteins that bind to DNA. It has been suggested that an increased level of the effector could arise from exonuclease V activity (Eisenstark, 1977). Emmerson & West (1978) have put forward evidence to suggest that an increased effector level results from an idling reaction of DNA polymerase III at a stalled replication fork, due to an unexcised pyrimidine dimer, or a malfunctioning replication complex. DNA polymerase III has been shown to be essential for mutagenesis after UV (Bridges *et al.*, 1976; Bridges, 1978). In addition a revertant of *pol* C that is unable to perform mutagenic repair but is able to carry out error free repair has been isolated (Bridges, 1978). The substrate for repair seems to be certain types of SS gaps in DNA and there appears to be a fraction of the POL III replication complex that is especially required for inducible mutagenic repair. Bridges (1978) has suggested that an inducible cofactor is required to enable Pol III to polymerase in an error prone way. Radman (1977) has proposed that daughter strand gaps are kept open by 3'5' exonuclease activity of a polymerase (Pol III?) which repeatedly excises any bases inserted opposite a damaged base in the template strand. Inducible repair might operate by inhibiting this exonuclease activity thus permitting further chain elongation. Mutagenic repair shows a clear delay of 15 - 20 m and is inhibited by chloramphenicol indicating that protein synthesis is required (Bridges, 1978). Evidence suggests that an inhibitor of 3' -5' exonuclease activity allows the polymerase to proceed and it is possible that the *uvr* D gene product might act as the cofactor (Kushner *et al.*, 1978). This has been shown schematically in Fig. 10.5. The presence of inducible editing inhibitors in UV irradiated cells would render all DNA synthetic pathways using susceptable polymerases error prone so that it is probable that no one pathway can be considered as the error prone or the mutagenic pathway.

The *lex* gene product has been shown to regulate the *rec* A gene function and several models have been proposed to explain its function (Witkin, 1976; Eisenstark, 1977; Bailone *et al.*, 1979). Gudas and Pardee (1975) were the first to suggest that the *rec* A product together with the products of DNA degradation are required to remove a repressor coded for by the *lex* gene, from the *rec* A operon. McEntee (1978) isolated a protein (85 000 molecular weight) which is the *lex* gene product and his studies on the regulation of *rec* A function are consistant with negative control of *rec* A expression by the *lex* product. This is shown schematically in the model shown in Fig. 10.5. It appears that low levels of *rec* A protein, normally present in the cell, are sufficient to allow recombination processes to occur, but only when the *lex* inhibitor has been removed is there sufficient *rec* A protein synthesis to enable inducible (SOS) processes to occur in the cell. Although other models of *lex* function have been proposed, the Gurdas & Pardee model (1975) has the most experimental support.

10.2 METHODS

10.2.1 PHOTOREACTIVATION

Growth and irradiation procedures were carried out as described in 7.2.2 & 7.2.3 All photoreactivation experiments were performed in a darkroom using an orange safe light. Photoreactivation treatments consisted of exposing UV irradiated cell suspensions to visible light for 20 m at room temperature. The source of illumination used was a tungsten halogen lamp (Zeiss) fitted with a Corning 0-52 filter which absorbed all radiation below 350 nm. The lamp was calibrated using a YSI - Kettering 65A radiometer and a fluence of 1 000 J m^{-2} s^{-1} was used in all the experiments. Additional experiments were also conducted in which cell suspensions in holding buffer were exposed to fluorescent light during a 12 h period. The number of viable cells after photoreactivation treatments was determined using the methods described in 7.2.4 and 8.2.4.

10.2.2 EXCISION OF PYRIMIDINE DIMERS

The percentage of ^{3}H-thymidine which occurred as dimers was assayed in both the acid insoluble and the acid soluble fractions, in unirradiated cells and cells irradiated under aerobic conditions with fluences of 30, 34 and 38 J m^{-2}. Assays were carried out immediately after irradiation, and after 2,5 h liquid holding under both anaerobic (AS) and aerobic (RS) conditions. The assay of cyclobutyl pyrimidine dimers was carried out according to the method already described (7.2.5).

10.2.3 ISOLATION OF UV RADIATION SENSITIVE MUTANTS

Four methods were used in attempts to isolate radiation sensitive mutants in *B. fragilis*. The first method did not involve any selection technique and consisted only of subjecting the cells to a chemical mutagenic treatment (as described in 2.2.7) and then testing the surviving colonies for increased sensitivity to UV radiation. Individual colonies were either transferred directly onto a test plate or were first subcultured into broth before testing. The test plate consisted of a BHI agar plate onto which a grid was ruled. The individual isolates to be tested were streaked across the surface of the plate, using a very fine platinum wire, so as to give a uniform streak of cells across the test plate. Fifteen isolates and a control could be tested by this method on each plate. The plate was then placed in a holder which was fitted with a shutter, which allowed fixed segments of the streaked colonies to be exposed to increasing fluences of UV radiation. One segment of the plate was protected from exposure to UV radiation and acted as a control for growth. UV survival curves were determined on any isolates which appeared to show increased sensitivity to UV radiation.

A second method used to screen for sensitive mutants, consisted of diluting and plating an overnight culture of chemically mutagenized cells onto BHI plates so as to give *c*. 500 c.f.u. per plate. The plates were incubated until very small colonies were just visible (18 h). These colonies were then replica plated onto a second plate and irradiated with a fluence of 35 J m^{-2} to give *c*. 10^{-3} survivors. Both the master plate and the replica plate were reincubated for a further 24 - 48 h and the colony formation on the two plates was compared. Any colonies which were absent on the replica plate were picked from the master plate and tested for UV sensitivity.

The third method used to try and isolate UV sensitive mutants was a modification of the method developed by Howard-Flanders & Theriot (1962) which is described in 9.2.3.

The fourth method employed was a modification of the penicilin selection technique used for obtaining DNA repair mutants in *E.coli* reported by Sancar & Rupert (1978, 1979). *B. fragilis* cells were mutagenized using the method of Van Tassell & Wilkins (1978) described in 2.2.7 . After the EMS treatment, cells were washed and grown up in BHI overnight (15 h) . The overnight culture was diluted 1:10 in the same medium and grown at 37°C to a density of *c.* 5 x 10^8 ml^{-1} cells. The cells were washed and diluted 1:10 in RB and irradiated with a fluence of 22 J m^{-2} (10^{-1} survivors). The irradiated cells were then centrifuged and resuspended in complete or minimal penicillin-clavulanic acid selection medium. In one experiment cells were held in buffer for 2,5 h before being resuspended in the selection medium. The cells were then grown for 1,5 or 2,5 h at either 37°C or 42°C and then subjected to osmotic shock by resuspending in water at 50°C for 15 m. Samples were plated to determine the cell survival. Cell suspensions were then centrifuged and resuspended in the selection medium and allowed to grow at 37°C to a titre of 5 x 10^8 ml^{-1} cells. A second cycle of UV irradiation, penicillin treatment and osmotic shock was repeated and in some experiments a third cycle was also carried out. Cultures from the final cycle of penicillin treatment were diluted and plated onto BHI plates which were incubated anaerobically at 37°C for 48 h. Individual colonies were picked and tested for UV sensitivity using the shutter technique described above.

10.2.4 ISOLATION OF UV RADIATION RESISTANT MUTANTS

Two methods were used to screen for UV resistant mutants of *B.fragilis* In the first method *B.fragilis* cells were subjected to mutagenic treatments as described in 2.2.7 and then irradiated with high fluences of UV radiation (40 - 80 J m^{-2}) either while cells were in suspension or after plating onto the surface of BHI agar plates. Surviving colonies were then tested for increased resistance to UV radiation using the shutter technique.

The second method was based on the technique used by Witkin (1946) to isolate the *E.coli* B/r mutant. Mutagenized *B. fragilis* cells were grown overnight and diluted so as to give between 10^3 and 3×10^3 c.f.u. per plate. The plates were then irradiated with varying fluences to give *c.* 10^{-1}, 10^{-2} and 10^{-3} survivors and incubated for 4 - 6 h at 37°C. The plates were then reirradiated with high fluences (40, 60 or 80 J m^{-2}) and the plates were incubated for 48 h at 37°C. Any surviving colonies were tested for increased radiation resistance using the shutter technique.

10.2.5 COMPARISON OF THE NUMBER OF RIFAMPICIN RESISTANT MUTANTS IN STATIONARY PHASE CULTURES BEFORE AND AFTER MUTAGENIC TREATMENT.

The number of rifampicin resistant mutants present in a population of stationary phase *B.fragilis* cells was measured before and after mutagenic treatment under aerobic and anaerobic conditions. Stationary phase cell suspensions (*c.* 10^9 c.f.u. ml^{-1}) were irradiated with low fluences of UV radiation so as to give *c.* 10^{-1} survival (*c.* 10^8 c.f.u. ml^{-1}). Unirradiated cells from the same population were diluted 1:10 to give (*c.* 10^8 c.f.u. ml^{-1}) and the irradiated and diluted cell suspensions were plated onto BHI agar plates containing 20μg ml^{-1} rifampicin. In addition the number of viable cells in the different samples were determined by plate counts. The number of rifampicin resistant colonies present on 50 plates indicated the number of mutants present in 5 ml of cell suspension containing *c.* 5×10^8 c.f.u.

10.3 RESULTS

10.3.1 PHOTOREACTIVATION

B. fragilis cell suspensions were irradiated in the dark with increasing fluences of UV radiation and then held in buffer for 20 m with and without photoreactivation treatment, and the surviving fraction of cells determined. Similar experiments were conducted where the cell suspensions were given an extra 20 m liquid holding period after irradiation and before the photoreactivation treatment was given. In all experiments there was no difference in the number of surviving cells (Fig. 10.6). The effect of visible light on LHR was also investigated. Time course viability curves were determined for cells irradiated with a fluence of 30 J m^{-2} in the dark and then subjected to different photoreactivation treatments during the 12 h liquid holding period. Similar increases in viability of irradiated cells held in RB under aerobic conditions occurred irrespective of whether the cells were held in the dark, held in the light (fluorescent light), or subjected to a 20 m photoreactivation treatment immediately after UV irradiation and then held in the dark for the remainder of the holding period (Fig. 10.7). No increase in the viability of irradiated *B. fragilis* cells held under anaerobic conditions occurred, irrespective of whether cells were held in the dark or light. No difference in viability was obtained when plates were incubated in the light (fluorescent light) or in the dark after any of the treatments.

10.3.2 EXCISION OF PYRIMIDINE DIMERS

The number of ^{3}H-thymine dimers in the DNA of irradiated *B.fragilis* cells was assayed immediately after irradiation and after 2,5 h liquid holding under both anaerobic and aerobic conditions. In

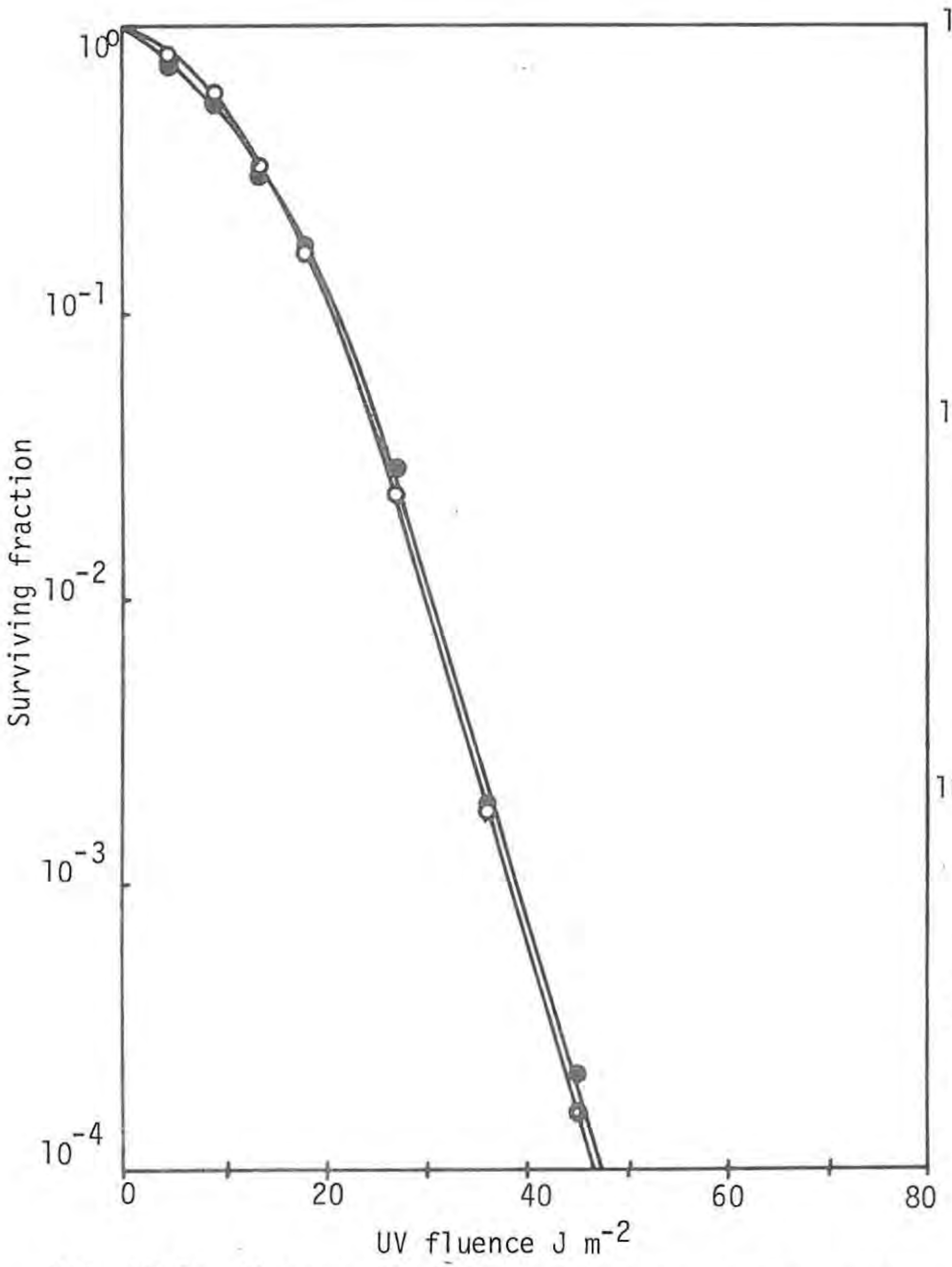


FIG. 10.6: Absence of photoreactivation in *B.fragilis* after UV irradiation. Survival of irradiated exponential phase cells, given a 20 min photoreactivation treatment (o), held in the dark for 20 min (●).

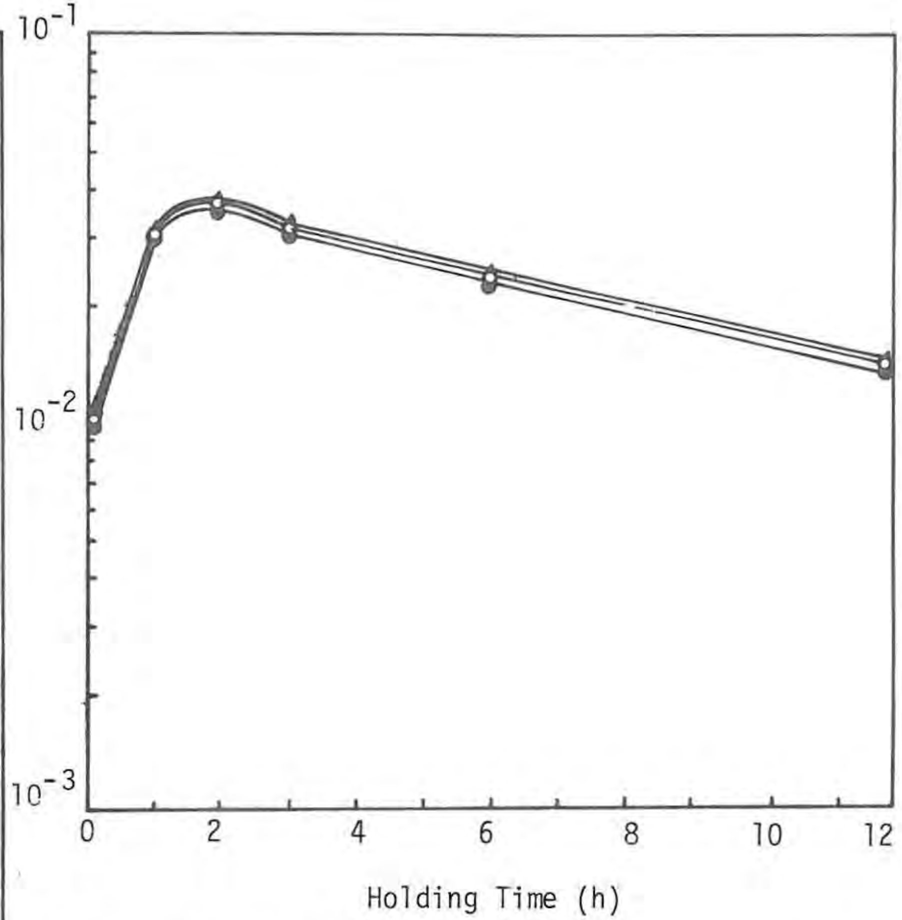


FIG. 10.7: The effect on the time course viability curves of exponential phase cells irradiated with a UV fluence of 30 J m^{-2} in the dark and held in the light (o), held in the dark (●), and given a 20 min photoreactivation treatment and held in the dark (▲).

addition the DNA of unirradiated cells was assayed as a control. Both the acid insoluble fractions (DNA fractions) and the acid soluble fraction (nucleotide fraction) were assayed for the presence of ^{3}H-thymine dimers. This experiment was repeated on 4 occasions in an attempt to obtain as high a level of ^{3}H-thymine in the assay samples as possible. However, when cell suspensions containing more than 5 x 10^{7} c.f.u. ml^{-1} were used, interference and smearing occurred in the chromatograms causing a loss in definition. The best results were obtained with less dense cell suspensions even though relatively low ^{3}H-thymine counts were obtained (20 000 to 35 000 c.p.m.). The results obtained for one assay are shown (Fig. 10.8). The acid insoluble or soluble fractions of the unirradiated DNA did not contain measurable quantities of dimers. In cells irradiated under aerobic conditions and assayed immediately *c.* 0,55% of the ^{3}H-thymine was found to occur as dimers in the acid insoluble fraction while only 0,1% was found in the acid insoluble fraction. In irradiated cells assayed after a 2,5 h liquid holding period under aerobic conditions, the percentage of ^{3}H-thymine occurring as dimers in the acid insoluble fraction showed a marked decrease (0,15%), and a corresponding increase in the percentage ^{3}H-thymine occurring as dimers was found in the acid soluble fraction (0,4%). This indicated that a movement of thymine dimers from the DNA into the cytoplasm or periplasmic space had probably occurred. In cells held under anaerobic conditions for 2,5 h a similar trend was observed but a higher percentage of ^{3}H-thymine dimers remained in the acid insoluble fraction (0,2%) and a lower proportion occurred in the acid soluble fraction (0,25%). The results obtained in the

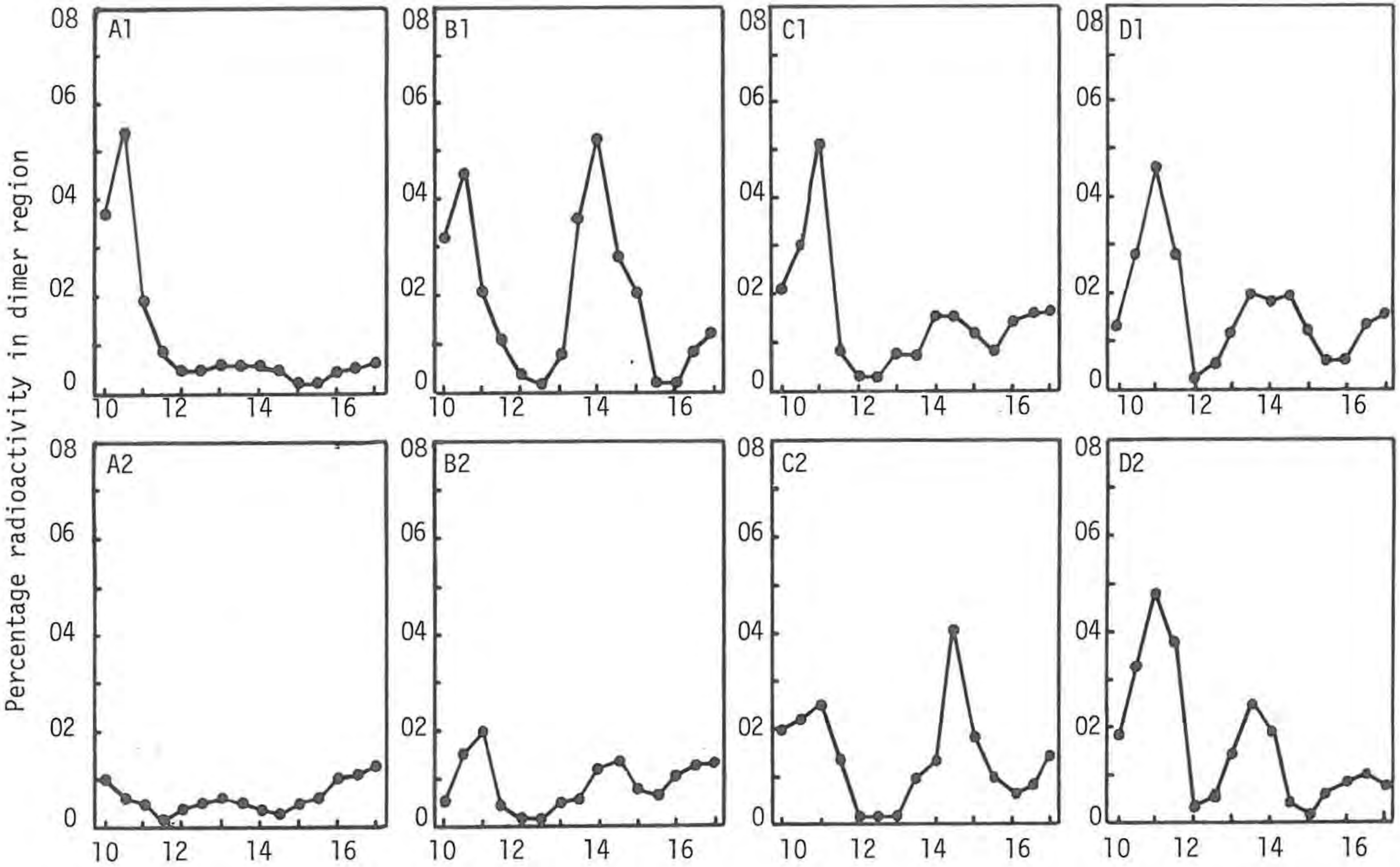


FIG. 10.8: The percentage of thymine-containing pyrimidine dimers induced in the DNA of *B.fragilis* unirradiated cells, acid insoluble fraction (A1) and acid soluble fraction (A2). Cells irradiated under aerobic conditions (20 J m^{-2}) assayed immediately, acid insoluble fraction (B1), acid soluble fraction (B2). Cells assayed after 2,5 h in RB (aerobic), acid insoluble fraction (C1), acid soluble fraction (C2). Cells assayed after 2,5 h in AB (anaerobic), acid insoluble fraction (D1), acid soluble fraction (D2).

other assay showed a similar pattern. These results suggest that although some dimers are removed under anaerobic conditions the disappearance of dimers from the DNA was not as marked as the removal of dimers under aerobic conditions.

10.3.3 THE ISOLATION OF UV RADIATION SENSITIVE AND RESISTANT MUTANTS OF *B. FRAGILIS*

Attempts to isolate mutants of *B. fragilis* which showed either increased or decreased sensitivity to UV radiation did not meet with much success. Using the technique of Sancar & Rupert (1979) over 1 000 individual colonies were isolated and tested for increased sensitivity to UV radiation. Of these, only 2 isolates appeared to show any marked increase in sensitivity and on further testing neither proved to be deficient in excision repair. The first mutant showed only a small increase in sensitivity (DEF 1,14 at 1% survival) and was able to carry out LHR (Fig. 10.9). The second mutant was interesting as it appeared to show quite a marked increase in sensitivity on the plate assay, but when the survival curve for this mutant was determined it showed an increase in survival and LHR (Fig. 10.9). This mutant showed a very marked retardation in its rate of growth which appeared to account for the difference in sensitivity observed under the two different conditions. The apparent sensitivity on the plate assay was due to the very slow rate of colony growth giving a false impression of UV sensitivity. However, when the survival curve was determined the slow growth rate allowed more efficient repair to occur (in much the same way as MMR), leading to increased survival (Fig. 10.9). As neither of the mutants appeared to be repair deficient, they were not investigated further. No true UV sensitive or resistant mutants

TABLE 10.2:

THE NUMBER OF RIFAMPICIN RESISTANT MUTANTS IN 10^8 c.f.u. ml^{-1} STATIONARY PHASE CELL POPULATIONS BEFORE AND AFTER MUTAGENIC TREATMENT.

MUTAGENIC TREATMENT	EXPT I	EXPT II	EXPT III
Untreated cells	0,6	0,2	1
Irradiated under aerobic conditions (10^{-1} survival)	0,2	0,6	-
Irradiated under anaerobic conditions (10^{-1} survival)	20	16	-
EMS treatment (Procedure partially aerobic)	-	-	0,2

KEY: - not done

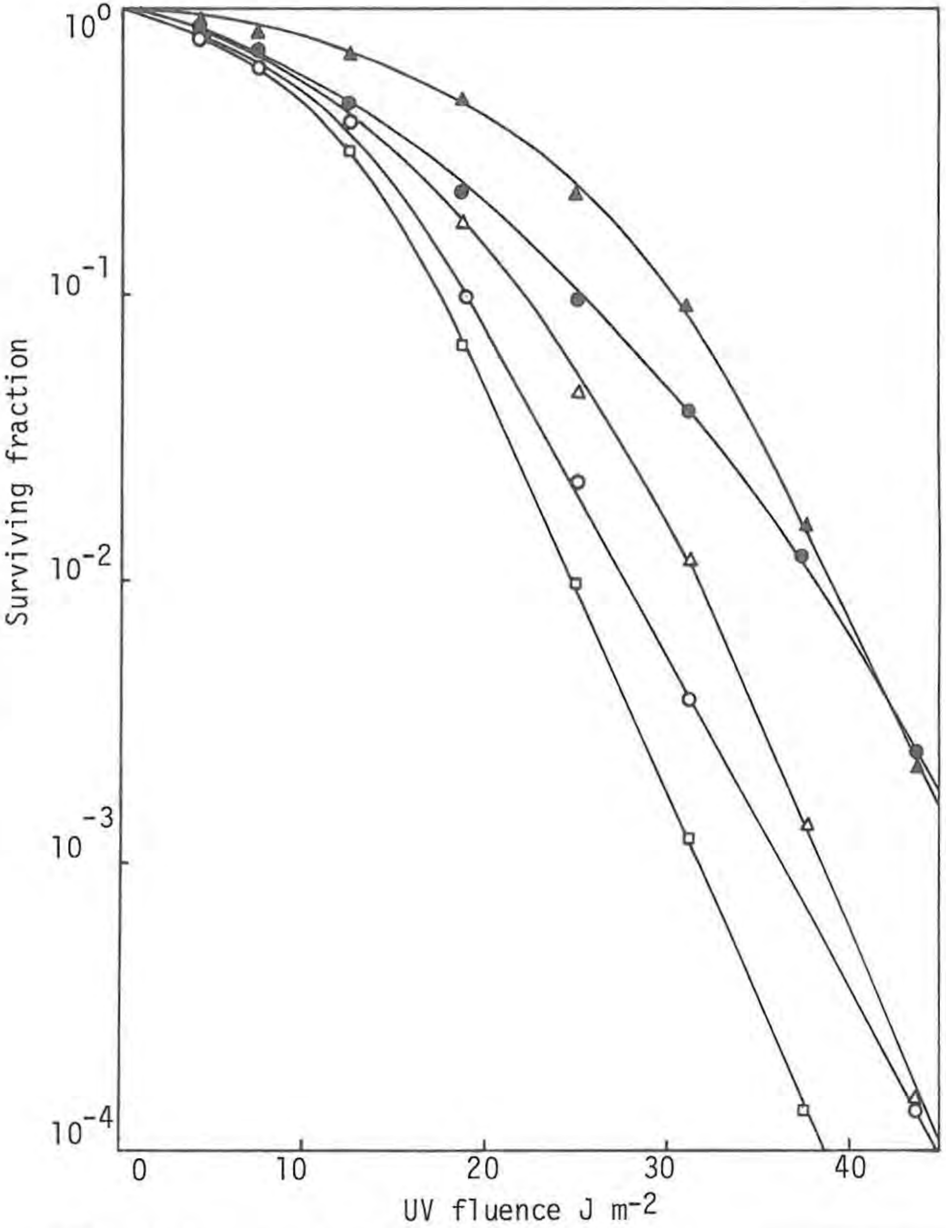


FIG. 10.9: Survival curves of exponential phase wild-type and mutant *B.fragilis* cells irradiated aerobically and plated immediately (o△), and held for 2,5 h aerobically (RS) (●▲). WT *B.fragilis* (o●), UV mutant 1 (□), UV mutant 2 (△▲).

were obtained from the many hundreds of colonies which were isolated using the various techniques.

10.3.4 RIFAMPICIN RESISTANT MUTANTS IN STATIONARY PHASE CULTURES BEFORE AND AFTER MUTAGENIC TREATMENT

The number of rifampicin resistant mutants in stationary phase populations were determined before and after UV irradiation under anaerobic and aerobic conditions. In 2 aerobic UV induction experiments the number of rifampicin resistant mutants before and after UV irradiation did not appear to differ significantly (Table 10.2). However, cells irradiated under anaerobic conditions did appear to show a significant increase in the number of rifampicin mutants (Table 4.2). In a further aerobic induction experiment with EMS there was no significant increase in the number of rifampicin mutants.

10.4 DISCUSSION

The results obtained from the photoreactivation experiments indicate that photoreactivation repair is absent in *B. fragilis* and the presence or absence of light has no effect on survival after UV irradiation. Although photoreactivation has been shown to be a very widely occurring phenomenon (Rupert, 1975), the absence of this mode of repair in *B. fragilis* is in keeping with the ecological niche occupied by an obligate anaerobe. In addition to *B. fragilis*, photoreactivation has also been shown to be absent in a number of other bacteria including *Haemophilus influenzae*, *Diplococcus pneumoniae*, *Bacillus subtilis*, *Micrococcus luteus* and *Micrococcus radiodurans* (Rupert, 1975). It is also interesting to note that photoreactivation is absent in *E.coli* when the cells are grown under anaerobic conditions (Tyrrell, 1973). Photoreactivation enzymes have been found to occur in a wide range of species, which in the bacteria include members of the mycoplasmas and actinomycetes. In eukaryotic organisms, photoreactivation enzymes have been found in members of all major groups of micro-organisms, plants and animals, including man (Rupert, 1975). Although its most obvious biological function would appear to be the repair of damage caused by solar irradiation. The presence of the enzyme in animal and plant tissue with no likelyhood of exposure to either UV radiation or visible light casts some doubt on this hypothesis.

Although *B.fragilis* lacks photoreactivation, it does possess an efficient dark repair mechanism. The results obtained from the cyclobutyl pyrimidine dimer assays, indicate that dimers are produced in more or less the same numbers after irradiation with the same fluence of UV radiation under both anaerobic and aerobic conditions. The percentage of dimers in the acid insoluble fraction was found to decrease after LHR had occurred under aerobic conditions and a corresponding increase in dimers was found in the acid soluble fraction, suggesting that dimers were excised from the DNA during LHR and accumulated either in the cytoplasm or the periplasmic space. Similar events are known to occur in *E.coli* as a result of excision repair processes (Setlow & Carrier, 1964). When irradiated *B.fragilis* cells were held in non-nutrient buffer under anaerobic conditions some of the dimers also appeared to be excised from the DNA but not as efficiently as they were under aerobic conditions.

The results obtained from the thymine dimer assay experiments indicate that *B. fragilis* possesses a repair mechanism capable of excising dimers from the DNA of irradiated cells, and that this repair mechanism is able to operate under both anaerobic and aerobic conditions to effect repair. The results also show that the recovery of cells held in buffer under aerobic conditions is brought about, at least in part, by the excision of thymine dimers from the DNA. The evidence obtained from the thymine dimer assays, coupled with the evidence obtained from the recovery experiments (reported in Chapter VIII) indicates that *B. fragilis* posesses an excision repair system which closely simulates that found in *E.coli* and other organisms. The repair process involved in LHR and other recovery phenomenon in *B.fragilis* appears similar to the nutrient-independent short patch excision repair pathway in *E.coli*, in that it is nutrient independent, temperature dependent, and is blocked by the action of caffeine and acriflavine. The results obtained for the experiments using inhibitors of excision repair suggests that this repair mechanism is involved in the increased survival which occurs in cells irradiated under anaerobic conditions This repair pathway also appears to function more efficiently in the absence of rapid growth and DNA synthesis, as does the nutrient independent excision repair system in *E.coli* (Swenson, 1976). The pathway also appears to be at least partially responsible for the increased survival encountered in stationary phase *B.fragilis* cells and for the increased survival after MMR. The difference in the survival of *B.fragilis* cells irradiated under anaerobic and aerobic conditions indicate that repair processes operate more effectively under anaerobic conditions. This could be interpreted as either indicating that the 'normal' excision repair pathway (the nutrient independent pathway) operates more efficiently under anaerobic conditions, or that another pathway (or pathways) becomes active under anaerobic conditions and acts to enhance the repair capabilities of the cell. The results obtained from the LHR experiments show that the first suggestion is unlikely, as liquid holding of irradiated cells in a non-nutrient buffer under anaerobic conditions, should have resulted in a greater level of recovery than occurs when cells are held under aerobic conditions. As the opposite was found to be the case, the second suggestion appears to provide a better explanation. It is possible to interpret the results obtained from the UV survival experiments and the LHR experiments as indicating that a second pathway or mode of repair exists in *B.fragilis* which is only able to function under strictly anaerobic conditions. Under conditions of growth (nutrient conditions) this additional mode (or modes) of repair leads to enhanced survival under anaerobic conditions. However, when *B.fragilis* cells are held in a non-nutrient buffer under anaerobic conditions the two modes of repair interact in a way that both beneficial and deleterious processes take place which result in no net increase in cell viability. The lack of LHR in *B.fragilis* under anaerobic conditions shows some similarities to the process which occurs in irradiated repair competent *E.coli* K-12 cells when held in non-nutrient buffer (Tang *et al.*, 1979).

In order to characterize repair processes in *B.fragilis* further, it is essential to obtain radiation sensitive mutants which are repair deficient. In particular, mutants which are unable to carry out nutrient independent excision repair would be of great value. The isolation of such mutants would almost certainly provide a greater understanding of the role of this repair process in cell survival following UV irradiation and would help to characterize the LHR and MMR phenomena further. In addition, mutants would be useful in clarifying the role of excision repair processes in survival and HCR of irradiated phages. The isolation of mutants which were deficient in excision repair would also allow an investigation of post-replication repair processes in *B. fragilis*, as any investigation in repair competent cells would be extremely difficult. However, all attempts to isolate such mutants have been unsuccessful.

In *E.coli* both the nutrient dependent excision repair pathway (the long patch pathway) and the post-replication repair pathway depend on a functional *rec* A gene product. Both pathways differ from the nutrient independent excision repair pathway (short patch pathway) in that they appear, at least to some extent, to be both error prone and inducible. As such they appear to be responsible for the mutagenic effects of UV radiation and a number of other physical and chemical mutagenic agents which inflict damage on the cells' DNA.

In *B.fragilis* there is some evidence which suggests that some repair processes might exist which also involve a *rec* A type of cell product. In *E.coli* both UVR and MR of certain phages is dependent on *rec* A function. In *B.fragilis* both processes occur but at somewhat lower levels of efficiency than in *E.coli*. However, the most interesting feature of these two reactivation processes is that they only occur under totally anaerobic conditions. This suggests that a *rec* A type of function in *B.fragilis* may only occur in the complete absence of oxygen. These findings also lend support to the suggestion that a second mode of repair may operate in *B.fragilis* only under anaerobic conditions. The fact that in *E.coli* K-12, LHR only occurs in *rec* A^- cells suggests that a similar situation might exist in *B.fragilis* where under anaerobic conditions *B.fragilis* cells behave like repair competent *rec* A^+ *E.coli* K-12 cells and under aerobic conditions they function like *rec* A^- cells.

The results obtained from the comparisons of the number of rifampicin mutants present in stationary phase populations before and after UV irradiation under aerobic and anaerobic conditions are probably of limited value. However, they do suggest that the induction of mutation by UV radiation might also only occur under strictly anaerobic conditions where a *rec* A type of function is operational. It would be of interest to investigate this aspect further by determining the actual rate of mutation under the two conditions. In *E.coli* only cells which are *rec* A^+ show UV mutagenesis. The *rec* A gene product is necessary both for the mutagenic and the other UV inducible functions such as prophage induction and UVR in *E.coli*. It can be suggested therefore, that under strictly anaerobic conditions, irradiated *B.fragilis* cells are able to function as a *rec* A^+ type of cell which can carry out inducible functions such as 'SOS' repair leading to mutagenesis, UVR and MR. However, under conditions where the cells have been exposed to oxygen, this *rec* A type of function is inhibited and the cell is unable to carry out any of the inducible functions or repair processes which require the functional *rec* A gene product.

CHAPTER XI

SUMMARY AND CONCLUSION OF PART II

The investigation reported in the recond part of this thesis was conducted on a single strain of *B.fragilis*. Although there is no reason to suspect that the physiological responses to UV radiation of this particular organism are not typical of the group as a whole, caution should be exercised in accepting these findings as being applicable to all strains of *B.fragilis*. Initial studies of the physiological responses of *B.fragilis* to UV radiation have shown that both the recovery and repair processes occurring in *B.fragilis* after UV radiation differ in a number of ways from those known to occur in *E.coli* and other aerobic species of bacteria.

The observation that *B.fragilis* was more sensitive to UV radiation when irradiated under aerobic conditions appears to be unique. In all other orgnisms investigated so far survival following UV irradiation has been shown to be independent of oxygen. The assay of pyrimidine dimers formed in *B.fragilis* during irradiation under anaerobic and aerobic conditions showed no significant differences in the amount of dimer formation. These results indicated that the decrease in survival of cells irradiated under aerobic conditions was not due to an increase in the amount of damage sustained by the DNA. The decreased survival of cells irradiated under aerobic conditions was reflected by a decrease in the size of the shoulder of the survival curve. This shoulder was found to disappear almost completely when irradiated cells were plated in the presence of caffeine, indicating that the difference in survival was due to differences in the repair capabilities of the cells.
A decreased repair potential also appeared to be responsible for the

decrease in survival observed in exponential phase cells.

Filament formation was found to occur in *B.fragilis* cells after UV irradiation, but this phenomenon appeared to differ from the inducible filament formation which occurs in *E.coli* B and K-12 *lon* cells. In *B.fragilis* the formation of filaments was found to be fluence dependent and some septum formation occurred within the long filaments. Filament formation was also affected by holding, and growth conditions following irradiation. Although there did not appear to be any direct relationship between filament formation and the other recovery phenomena observed, further study is required.

The observation that LHR in *B.fragilis* only occurred in cells which have been held under aerobic conditions, also seems to be unique. At first sight, this appears to be a paradox as recovery only occurred under conditions of reduced repair potential. However, in *E.coli* LHR also only occurs in strains which have a reduced repair capability. Where the full repair capability is present LHR in inhibited. The results obtained in this study indicate that LHR in *B.fragilis* was largely or solely due to excision repair. Maximum LHR occurred in non-nutrient holding media and the recovery effect was greatest in exponential phase cells. The recovery process was found to be temperature dependent, but independent of nutrients and protein synthesis. Caffeine and acriflavine inhibited LHR and both chloramphenicol and nalidixic acid produced a decrease in the amount of LHR. Although LHR in *B.fragilis* shows many similarities to the process which occurs in certain strains of *E.coli*, it does differ in a number of ways. In *B.fragilis* the LHR only occurred under aerobic conditions, it was not inhibited by yeast extract and some recovery apparently continued to occur after the cells had been plated out onto nutrient media following liquid holding treatment. Minimal medium recovery was also found to occur in *B.fragilis* and the effects of LHR followed by MMR were found to be cumulative indicating that excision repair functions most efficiently under conditions of reduced or suboptimal growth.

The survival curves of irradiated *B.fragilis* phages showed either exponential inactivation kinetics or a slight shoulder. However, the determination of phage inactivation by UV radiation was complicated by variations in the e.o.p. of the phages which were affected by the growth phase and condition of the host cells. All the *B.fragilis* phages tested were more resistant to UV radiation than *E.coli* T phages. When phage infected host cells were plated onto media containing caffeine a slight decrease in phage survival occurred. These results suggested that HCR brought about by caffeine-sensitive host cell excision repair, did not appear to occur to any great extent in any of the *B.fragilis* phages tested. The isolation of excision repair deficient mutants of *B.fragilis* appears to be essential for the further elucidation of HCR in this strain. In the 2 phages examined some UVR was found to occur under anaerobic conditions, but not under aerobic conditions, indicating that the host cell repair mechanism responsible for UVR was inhibited in the presence of oxygen. A similar result was obtained in the MR experiment where some MR was observed under anaerobic conditions but not under aerobic conditions. In *E.coli* both reactivation processes are known to require a functional *rec* A gene product.

The repair of UV-damaged DNA by photoreactivation was shown to be absent in the *B.fragilis* strain examined. Although photoreactivation has been found to occur in all the major groups of organisms, there are a number of other species of bacteria which also lack photoreactivation.

This strain was found to possess an excision repair system. Pyrimidine dimers formed in the DNA during UV irradiation were found to be excised from the DNA during LHR. Excision repair occurred under both anaerobic and aerobic conditions and was found to be similar to the short patch excision repair pathway in *E.coli* in that it was nutrient independent, temperature sensitive, and was inhibited by caffeine and acriflavine. Excision repair in *B.fragilis* appeared to operate most efficiently in the absence of rapid growth and DNA synthesis,and was largely or solely responsible for LHR and MMR in the strain examined. Excision repair also appeared to be responsible for bringing about a significant amount of the recovery in irradiated *B.fragilis* cells as evidenced by the presence of a shoulder in the survival curves. The shoulder disappeared almost completely when irradiated cells were plated onto agar in the presence of caffeine.

The increase in survival of cells irradiated under anaerobic conditions, seemed to be due to an increased efficiency of repair. It is not known whether this enhanced repair was due to the increased efficiency of a single mode of repair in the absence of oxygen, or whether a second mode or pathway of repair became operational under these conditions. The evidence from the LHR experiments however, suggested that a second mode of repair came into action. The results from the LHR experiments suggested that oxygen inhibits some aspect of "recombination repair" resulting in sensitization to UV and the expression of LHR. Further evidence for the inhibition of a *rec* A-like repair system by oxygen in *B.fragilis* was obtained from the UVR and MR experiments mentioned previously. The experiments conducted to assess the induction of mutagenesis following UV irradiation under anaerobic and aerobic conditions also indicated that inducible error-prone (SOS) repair only occurred under anaerobic conditions. All of these "*rec* A-dependent" processes in *B.fragilis* appeared to be extremely sensitive to oxygen, and exposure to small amounts of air were sufficient to abolish the processes.

The finding that *B.fragilis* apparently possess a *rec* A-like function which is inhibited by oxygen,has a number of implications for future studies on the genetics of this organism. The ability of *B.fragilis* to tolerate substantial exposure to oxygen without loss of viability has been extremely useful in studying this organism, as it has allowed many experaments to be performed partially or completely on the laboratory bench. However, it is obvious that any attempts to induce or study processes which are dependent on such '*rec* A'-type of functions are bound to fail if carried out under these conditions. In *E.coli*, processes dependent on *rec* A function, include chromosomal recombination after conjugation, transduction or transformation; long-patch excision repair; post replication repair; inducible error-prone repair; mutagenesis induced by some but not all chemical mutagens; the process of UVR and MR in some phages; the induction of prophages and possibly the induction of some bacteriocins. In the light of these findings it is apparent that many of the experiments reported in Part I of this thesis need to be reassessed as in virtually every case cells were exposed to oxygen at some stage during the procedure. It should be noted however, that even when experiments were performed under completely anaerobic conditions, the results from investigation of UVR and MR, and the induction of mutations after UV irradiation, indicated that '*rec* A' dependent effects in *B.fragilis* may be less pronounced than those occurring in aerobes. In addition, although some of the mutagenic agents used in this study are known to act independently of *rec* A function they did not appear to produce any significant increase in mutation frequency in *B.fragilis*. Both nitrous acid and frameshift-mutagens which are known to act independently of *rec* A function (Bernstein *et al.*, 1976; Drake & Baltz, 1976) were found to cause a rapid loss in viability with a low yield of mutants in *B.fragilis*.

If these preliminary findings on recombination function in *B.fragilis* prove to be correct then there would seem to be a need to reinvestigate a number of the phenomena which have already been studied. A study on the frequency of mutations under anaerobic and aerobic conditions, and further studies on inducible repair processes might provide information which would allow the induction of mutagenesis to be optimized in *B.fragilis*. Further studies on recombination processes with the aim of obtaining chromosomal recombination also seem called for. As do studies on induction processes aimed at inducing lysogenic phages in this group.

The isolation of repair-deficient mutants, particularly excision repair-deficient mutants (*uvr*) would allow an investigation of post-replication repair, and further characterization of excision repair dependent processes, such as survival, LHR, MMR and HCR. In addition such mutants would be extremely valuable in attempting to characterize any '*rec* A' dependent repair processes which might exist in *B.fragilis*.

APPENDIX I

CHARACTERISTICS OF *BACTEROIDES* STRAINS

Strain No	Species	Origin	Old Code	Phage sensitivity (P/R)	Bacteriocin production (B)	Bacteriocin sensitivity (S)	Colonial phase variation (V)
P1	*fragilis*	UN	F22	+++	-	-	±
PS1	*fragilis*	UN	C1	++++	-	+++	-
PS2	*fragilis*	UN	A1	++++	-	+++	+
PS3	*fragilis*	UN	F14	++	-	+++	+
PS4	*fragilis*	UN	F6	++	-	+++	-
PS5	*fragilis*	UN	F7	++	-	+++	
PS6	*fragilis*	UN	F8	++	±	+++	±
PS7	*fragilis*	UN	F13	++	±	+++	-
PS8	*fragilis*	UN	F15	++	-	+++	-
PB1	*fragilis*	UN	02	++++	++++	-	+
PB2	*fragilis*	UN	M1	+++	++++	-	±
PB3	*fragilis*	UN	F20	+++	++++	-	±
RB1	*fragilis*	UN	F16	+	++++	-	+
RB2	*fragilis*	UN	01	±	++++	-	+
RB3	*fragilis*	UN	F5	-	++++	-	-
RB4	*fragilis*	UN	FM1	-	++++	-	±
RB5	*fragilis*	UN	F2	-	++	±	+
RBS1	*fragilis*	VPI(USA)	F1	-	++	+++	-
RBS2	*vulgatus*	RU	R1	-	+++	++	±
RBS3	*vulgatus*	UN	R2	-	+++	++	+
RS1	*fragilis*	UN	F3	-	-	+++	+
RS2	*fragilis*	UN	F4	-	±	+++	-
RS3	*fragilis*	UN	F9	-	-	+++	±
RS4	*fragilis*	UN	F10	-	±	+++	+
RS5	*fragilis* (other)	UN	F01	-	-	+++	+
RS6	*fragilis* (other)	UN	F02	-	-	+++	±
RS7	*fragilis*	UN	S1	-	-	+++	-
RS8	*vulgatus*	UN	R3	-	-	+++	-
R1	*fragilis*	UN	F21	-	+	+	-
R2	*fragilis*	UN	F17	-	±	+	-
R3	*fragilis*	UN	F18	-	-	+	+
R4	*distasonis*	UN	D1	-	-	±	±
RV1	*fragilis*	UN	F11	-	-	-	++++
RV2	*fragilis*	UN	F12	-	-	-	++++
RV3	*fragilis*	UN	F19	-	-	-	++++
RV4	*thetaiotaomicron*	RU	T1	- * (sensitive to T phages).	-	-	++++

KEY:
Origin : UN University of Natal. RU Rhodes University
Phage Sensitivity : ++++ Sensitive to all - Not sensitive to any
Bacteriocin Production : ++++ Effective against all sensitives - No production
Bacteriocin Sensitivity : ++++ Sensitive to all bacteriocins - Not sensitive
Colonial Phase Variation : ++++ Marked phase variation - Very weak/no variation

NEW CODE FOR STRAINS

P = Phage Sensitive Strains
R = Phage Resistant Strains
V = Strains showing Strong Colonial Phase Variation
B = Bacteriocin Producing Strains
S = Bacteriocin Sensitive Strains

APPENDIX II

MEDIA AND SOLUTIONS

1. PREPARATION OF ANAEROBIC MEDIA AND SOLUTIONS

Pre-reduced and anaerobically sterilized media and anaerobic solutions were prepared according to the methods of Moore (1966) and Hungate (1969). Most of the oxygen was driven off by heating. The media and solutions were further reduced by the addition of cysteine hydrochloride or sodium thioglycollate. They were then flushed with a $CO_2 + H_2$ mixture and maintained in an anaerobic state in Astell roll tubes with tightly fitting rubber stoppers, Hungate tubes, or by storing in anaerobic jars or boxes with silica gel in muslin bags.

All sterilization was done by autoclaving at 121°C for 20 min unless otherwise stated.

2. GENERAL SOLUTIONS USED IN ANAEROBIC MEDIA

2.1 CYSTEINE STOCK SOLUTION (prepared freshly each time)

Cysteine hydrochloride	1,0 g
distilled H_2O	20,0 ml

Autoclaved.

1 ml 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 HEMIN-MENADIONE SOLUTION (H-M)

2.2.1 Menadione Stock Solution

Menadione	100 mg
95% ethanol	20 ml

Filter-sterilized and stored at 4°C

2.2.2 Hemin Stock Solution

Hemin	50 mg
IN NaOH	1 ml
Dist. H_2O	100 ml

Autoclaved and stored at 4°C.

2.2.3 H-M Solution

Menadione stock	1 ml
Hemin stock	100 ml

Stored at 4°C.

1 ml of H-M solution was added per 100 ml cooled medium prior to pouring to give a final hemin concentration of 5 $\mu g\ ml^{-1}$.

2.3 SODIUM CARBONATE STOCK SOLUTION

Na_2CO_3	20 g
Dist. H_2O	100 ml

Autoclaved.

2 ml of Na_2Co_3 stock was added per 100 ml medium to give a final concentration of 4 $mg\ ml^{-1}$.

2.4 RESAZURIN STOCK SOLUTION

Resazurin	20 mg
Dist. H_2O	100 ml

1 ml of Resazurin stock was added per 100 ml medium to give a final concentration of 2 $\mu g\ ml^{-1}$.

2.5 SEMI-SOLID ANAEROBIC INDICATOR

Sodium thioglycollate	0,25 g
di-sodium tetraborate (borax)	0,4 g
Agar	0,7 g
Dist. H_2O	100,0 ml
Resazurin stock soln.	1,0 ml

The indicator was steamed for 30 min, dispensed into roll tubes in 5 ml amounts and gassed immediately.

3. GENERAL ANAEROBIC NUTRIENT MEDIA

3.1 BEEF LIVER MEDIUM FOR ANAEROBES (from the American Type Culture Collection Catalogue)

Beef liver (cut up into small pieces)	500,0 g
Tap water	1,0 l

The beef liver was soaked overnight in the fridge, the fat skimmed off the top, autoclaved for 10 min at 121°C and filtered through cheese cloth. The meat was saved. To the liquid was added:-

Peptone	10,0 g
K_2HPO_4	1,0 g

The pH adjusted to 8,0, filtered through filter paper and the volume made up to one litre with tap water. A small amount of $CaCO_3$ was placed in each tube, 1,5 cm of meat was added, covered with broth to a total depth of 5 cm, and autoclaved.

3.2 BRAIN HEART INFUSION BROTH

BHI	3,7 g	18,5 g
Yeast extract	0,5 g	2,5 g
Sodium thioglycollate	0,11 g	0,55 g
Na_2CO_3	0,4 g	2,0 g
Dist. H_2O	100,0 ml	500,0 ml
Resazurin Stock soln.	1,0 ml	5,0 ml

The medium was steamed for 30 min, 1,0 ml H-M Soln. was added per 100 ml, dispensed into Hungate tubes in 10 ml amounts, gassed immediately and autoclaved.

3.3 BRAIN HEART INFUSION AGAR

3.3.1 Plates

BHI	3,7 g	18,5 g
Yeast extract	0,5 g	2,5 g
Agar	1,5 g	7,5 g
Dist. H_2O	100,0 ml	500,0 ml

The medium was autoclaved, cooled and

cysteine stock soln.	1,0 ml	5,0 ml
Na_2CO_3 stock soln.	2,0 ml	10,0 ml
H-M soln.	1,0 ml	5,0 ml

were added prior to pouring.

3.3.2 Slopes

BHI	3,7 g	18,5 g
Yeast extract	0,5 g	2,5 g
Agar	1,5 g	7,5 g
Dist. H_2O	100,0 ml	500,0 ml

The medium was steamed for 30 min and

cysteine stock soln.	1,0 ml	5,0 ml
Na_2CO_3 stock soln.	2,0 ml	10,0 ml
H-M soln.	1,0 ml	5,0 ml

were added, medium dispensed into 10 ml amounts in Astell roll tubes, gassed, clamped, autoclaved and sloped.

3.3.3 Soft Agar

BHI	3,7 g
Yeast extract	0,5 g
Na_2CO_3	0,4 g
Dist. H_2O	100,0 ml

The medium was autoclaved and stored aerobically. It was boiled before use, cooled to 50°C in a waterbath and 1 ml H-M soln. added.

3.4 PEPTONE-YEAST EXTRACT MEDIUM (PY)

3.4.1 Salts Stock Solution

$CaCl_2$ (anhydrous)	0,2 g
$MgSO_4$	0,2 g
K_2HPO_4	1,0 g
$K\ H_2PO_4$	1,0 g
$NaH\ CO_3$	10,0 g
NaCl	2,0 g
H_2O	1000,0 ml

Each component was dissolved separately and then mixed in the above order, and stored over 10% chloroform at 4°C.

3.4.2 PYG Medium

Glucose	0,5 g
Peptone	1,0 g
Yeast extract	1,0 g
Salt stock solution	4,0 ml
Resazurin	1,0 ml
H_2O	100,0 ml

The medium was autoclaved and 1 ml of cysteine stock solution added, dispensed into 10 ml volumes in Hungate tubes and gassed.

4. SPECIAL NUTRIENT MEDIA

4.1 ANTIBIOTIC MEDIA

Erythromycin, chloramphenicol and rifampicin were dissolved in 95% alcohol, nalidixic acid was dissolved in 0,1 N NaOH. All the other antibiotics were dissolved in sterile distilled water. No cysteine was added to media incorporating antibiotics. Antibiotic solutions were incorporated into media at doubling dilutions for m.i.c. determinations.

4.1.1 Antibiotic Gradient Plates

Erythromycin (25 $\mu g\ ml^{-1}$), Rifampicin (100 $\mu g\ ml^{-1}$), Vancomycin (15 $\mu g\ ml^{-1}$), Chloramphenicol (60 $\mu g\ ml^{-1}$), Tetracycline (100 $\mu g\ ml^{-1}$), Nitrofurantoin (50 $\mu g\ ml^{-1}$), were incorporated into BHl agar. Ten ml aliquots were poured into petri dishes and allowed to solidify in a slanted position. Ten ml BHl agar was poured onto this to give a flat surface.

4.1.2 Antibiotic plates

Erythromycin (6,25; 12,5 and 25 $\mu g\ ml^{-1}$), Rifampicin (10 and 20 $\mu g\ ml^{-1}$), Vancomycin (5; 10 and 20 $\mu g\ ml^{-1}$) and Nitrofurantoin (10 and 20 $\mu g\ ml^{-1}$) were incorporated into BHl agar prior to pouring.

4.1.3 a) Penicillin - Clavulanic Selective Medium

Clavulanic acid stock solution:	0,001 $g\ ml^{-1}$
Penicillin stock solution:	12 500 $U\ ml^{-1}$

0,1 ml of each was added to 10 ml BHl broth to give final concentrations of 10 $\mu g\ ml^{-1}$ and 125 $U\ ml^{-1}$ respectively.

4.1.3 b) Supplemented Penicillin - Clavulanic Selective Medium

BHl broth	100 ml
Sucrose	12 g
$MgSO_4$	0,25 g

0,1 ml Clavulanic and penicillin stock solutions were added to 10 mls of medium.

4.2 MEDIA CONTAINING INHIBITORS

4.2.1 Caffeine plates

Caffeine (1 mg ml^{-1} final concentration) was added to BH1 agar prior to pouring.

4.2.2 Sodium Arsenite Plates

Sodium arsenite (100 µg ml^{-1} final concentration) was added to BH1 agar prior to pouring.

4.3 MEDIA FOR PHASE VARIATION

4.3.1 pH Plates

BH1	3,7 g
Yeast extract	0,5 g
Sodium thioglycollate	0,11 g
Dist. H_2O	50 ml

The medium was steamed for 30 min to reduce, 50 ml of a 0,2 M phosphate buffer was added (6.2.1). The pH was adjusted to the required pH, 1,5 g agar was added. The medium was autoclaved, cooled and 1 ml H-M solution was added prior to pouring.

4.3.2 BH1 Plates for Phase Variation

	A	B
BH1	3,7 g	3,7 g
Yeast extract	0,5 g	0,5 g
KCl	1,0 g	-
Agar	1,5 g	1,5 g
Dist. H_2O	90,0 ml	100,0 ml

The medium was autoclaved and

H-M Soln.	1,0 ml	1,0 ml
Cysteine stock soln.	1,0 ml	1,0 ml
Na_2CO_3 stock soln.	2,0 ml	2,0 ml
10% glucose	5,0 ml	-
10% sucrose	5,0 ml	-

were added prior to pouring.

4.3.3 BH1 Broths for Phase variation

BH1	3,7 g
Yeast extract	0,5 g
KCl	1,0 g
Sodium thioglycollate	0,11 g
Na_2CO_3	0,4 g
Dist. H_2O	90,0 ml
Resazurin stock soln.	1,0 ml

The medium was steamed for 30 min to reduce, 1 ml H-M solution and 5 ml each of 10% glucose and 10% sucrose were added. The medium was dispensed into 10 ml amounts in Hungate tubes, gassed and autoclaved.

4.3.4 Blood Agar Base Plates

	A	B
Blood agar base	4,0 g	4,0 g
KCl	1,0 g	-
H_2O	90,0 ml	100,0 ml

The medium was autoclaved and

	A	B
H-M solution	1,0 ml	1,0 ml
Cysteine stock soln.	1,0 ml	1,0 ml
Na_2CO_3 stock soln.	2,0 ml	2,0 ml
10% glucose	5,0 ml	-
10% sucrose	5,0 ml	-

were added prior to pouring.

4.3.5 Peptone Agar Plates

	A	B
Peptone	1,5 g	1,5 g
Tryptone	1,5 g	1,5 g
NaCl	0,5 g	0,5 g
Agar	1,5 g	1,5 g
KCl	1,0 g	-
H_2O	90,0 ml	100,0 ml

The medium was autoclaved and

H-M soln.	1,0 ml	1,0 ml
Cysteine stock soln.	1,0 ml	1,0 ml
Na_2CO_3 stock soln.	2,0 ml	2,0 ml
10% glucose	5,0 ml	-
10% sucrose	5,0 ml	-

were added prior to pouring.

4.3.6 Peptone Broth

	A	B
Peptone	1,5 g	1,5 g
Tryptone	1,5 g	1,5 g
NaCl	0,5 g	0,5 g
KCl	1,0 g	-
Sodium thioglycollate	0,11 g	0,11 g
Na_2CO_3	0,4 g	0,4 g
Dist. H_2O	90,0 ml	100,0 ml
Resazurin stock soln.	1,0 ml	1,0 ml

The medium was steamed for 30 min to reduce, and

	A	B
H-M soln.	1,0 ml	1,0 ml
10% glucose	5,0 ml	-
10% sucrose	5,0 ml	-

were added. The medium was dispensed into 10 ml amounts in Hungate tubes, gassed and autoclaved.

5. MINIMAL MEDIA (MM)

5.1 SOLUTIONS FOR MM

5.1.1 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 1000 ml with distilled water. Stored over 10% chloroform at 4^oC.

5.1.2 $FeSO_4$ $7H_2O$ Stock Solution

$FeSO_4.7H_2O$	0,04 g
Dist. H_2O	100,0 ml

One ml of the solution added to 100 ml medium to give a final concentration of 4 μg ml^{-1}.

Stored over 10% chloroform at 4^oC.

5.1.3 $(NH_4)_2$ SO_4 Stock Solution (6 mM)

$(NH_4)_2$ SO_4	0,1588 g
Dist. H_2O	200 ml

The solution was autoclaved and 5 ml added per 100 ml medium.

5.1.4 Glucose Stock Solution

Glucose	10 g
Dist. H_2O	100 ml

The solution was autoclaved and 5 ml added per 100 ml medium to give a final concentration of 5 mg ml^{-1}.

5.1.5 Vit B12 Stock Solution

A stock solution of 1 μg ml^{-1} was made up with sterile distilled water and 0,5 ml was added per 100 ml of medium to give a final concentration of 0,005 μg ml^{-1}.

5.2 LIQUID MINIMAL MEDIUM (Varel & Bryant, 1974)

Mineral soln.	5,0 ml	25,0 ml
$FeSO_4$ stock soln.	1,0 ml	5,0 ml
$(NH_4)_2$ SO_4 stock soln.	5,0 ml	25,0 ml
Resazurin stock soln.	1,0 ml	5,0 ml
Dist. H_2O	79 ml	395 ml
Sodium thioglycollate	0,11 g	0,55 g

The medium was autoclaved and

Glucose stock soln.	5,0 ml	25,0 ml
Vit B12 stock soln.	0,5 ml	2,5 ml
H-M soln.	1,0 ml	5,0 ml
Na_2CO_3 stock soln.	2,0 ml	10,0 ml

were added aseptically then dispensed into sterile Hungate tubes in 10 ml amounts, gassed immediately using sterile needles.

An adaptation to Varel and Bryant's minimal broth was made by Van Tassell and Wilkins (1978) by substituting 0,1 M phosphate buffer for Na_2CO_3.

5.3 MINIMAL AGAR

5.3.1 Varel and Bryant Minimal Agar

Mineral soln.	5,0 ml	25,0 ml
$FeSO_4$ stock soln.	1,0 ml	5,0 ml
$(NH_4)_2$ SO_4 stock soln.	5,0 ml	25,0 ml
Agar (BBL purified)	1,5 g	7,5 g
Dist. H_2O	79,0 ml	395,0 ml

The medium was autoclaved and

Glucose stock soln.	5,0 ml	25,0 ml
Vit B12 stock soln.	0,5 ml	2,5 ml
H-M soln.	1,0 ml	5,0 ml
Cysteine stock soln.	1,0 ml	5,0 ml
Na_2CO_3 stock soln.	2,0 ml	10,0 ml

were added prior to pouring.

5.3.2 VPI Minimal Agar (van Tassell & Wilkins, 1978)

VPI minimal agar was prepared by adding the essential components of Varel and Bryant's medium to *E.coli* minimal A medium.

A.	$(NH_4)_2\ SO_4$	1,0 g
	Na Citrate	0,25 g
	KH_2PO_4	3,5 g
	$K_2H\ PO_4$	4,0 g
	$MnCl_2 .4H_2O$	0,0005 g
	$MgCl_2 .6H_2O$	0,01 g

The salts were dissolved in 175 ml H_2O, the pH adjusted to 6,65, then autoclaved.

B.	$FeCl_3 .6H_2O$	0,0015 g
	$CaCl_2 .2H_2O$	0,015 g
	Agar (BBL purified)	7,5 g

The salts were dissolved in 250 ml H_2O by steaming for 30 min. The solution autoclaved, cooled to ± 60°C and added to soln A.

Glucose stock soln	50,0 ml
H-M soln	5,0 ml
Cysteine stock soln	5,0 ml
Na_2CO_3 stock soln	10,0 ml
Vit B12 stock soln	2,5 ml

were added prior to pouring.

5.4 SUPPLEMENTED MINIMAL MEDIA

5.4.1 Vitamin supplement (B-vitamin solution Varel & Bryant, 1974)

	Stock soln of vitamins	Vol. of stock added to make 100 mls of vitamin soln	Final concentration of vitamins in vitamin mixture
thiamin-hydrochloride	10 mg ml^{-1}	2 ml	200 μg ml^{-1}
calcium-D-pantothenate	10 mg ml^{-1}	2 ml	200 μg ml^{-1}
nicotinamide	10 mg ml^{-1}	2 ml	200 μg ml^{-1}
riboflavin	10 mg ml^{-1}	2 ml	200 μg ml^{-1}
pyridoxine-hydrochloride	10 mg ml^{-1}	2 ml	200 μg ml^{-1}

p-aminobenzoic acid	1 mg ml^{-1}	1,0 ml	10 μg ml^{-1}
biotin	1 mg ml^{-1}	0,25 ml	2,5 μg ml^{-1}
folic acid	1 mg ml^{-1}	0,25 ml	2,5 μg ml^{-1}
vitamin B12	1 mg ml^{-1}	0,1 ml	1,0 μg ml^{-1}

0,5% (V/V) B-vitamin solution was added to medium

5.4.2 Amino Acid Supplement

Amino acid stock solution

L-histidine-hydrochloride	50 mg
L-tryptophan	50 mg
Glycine	50 mg
L-tyrosine	50 mg
L-arginine-hydrochloride	100 mg
L-phenylalanine	100 mg
L-methionine	100 mg
L-threonine	100 mg
L-alanine	100 mg
L-lysine	150 mg
L-serine	150 mg
L-valine	150 mg
L-isoleucine	150 mg
L-proline	150 mg
L-aspartic acid	150 mg
L-leucine	200 mg
L-glutamic acid	448 mg

The amino acids were dissolved in 100 ml distilled water and added to the medium at a concentration of 20% (v/v).

5.4.3 Additional supplements

Casamino acid, casitone (Difco vitamin free), and tryptone stock solutions: 10 gm dissolved in 100 ml dist. H_2O. Two ml was added to 100 ml minimal broth to give a final concentration of 2 mg ml^{-1}.

Casitone/peptone stock solution: 5 gm of each were added to 100 ml dist. H_2O. Two ml was added to 100 ml minimal broth to give a final concentration of 1 mg ml^{-1} each.

5.5 SPECIALIZED MINIMAL MEDIA

5.5.1 a) Penicillin-Clavulanic Minimal Medium

0,1 ml penicillin and clavulanic stock solutions were added to 10 ml of minimal broth to give final concentrations of 125 U ml^{-1} and 10 μg ml^{-1} respectively.

5.5.1 b) Supplemented Penicillin-Clavulanic Minimal Medium

Minimal broth	100,0 ml
Casamino acid stock soln	2,0 ml
Sucrose	12,0 g
$MgSO_4$	0,25 g

0,1 ml of penicillin and clavulanic stock solutions were added to 10 ml of supplemented minimal broth.

5.5.2 Sulphanilamide Supplement Liquid Minimal Medium

Minimal medium supplemented with

Sulphanilamide	1000 μg ml^{-1}
Casamino acid	1000 μg ml^{-1}
Xanthine	25 μg ml^{-1}
5-Bromouracil	50 μg ml^{-1}
Uracil	2.5 μg ml^{-1}

5.5.3 Phase Variation Minimal Medium

	A	B
Mineral solution	5,0 ml	5,0 ml
$FeSO_4$ stock soln	1,0 ml	1,0 ml
$(NH_4)_2\ SO_4$ stock soln	5,0 ml	5,0 ml
KCl	1,0 g	-
Agar	1,5 g	1,5 g
H_2O	75,0 ml	85,0 ml
The medium was autoclaved and		
H-M soln	1,0 ml	1,0 ml
Cysteine stock soln	1,0 ml	1,0 ml
Na_2CO_3 stock soln	2,0 ml	2,0 ml
Vit B12 stock soln	0,5 ml	0,5 ml
10% glucose	5,0 ml	-
10% sucrose	5,0 ml	-

were added prior to pouring.

6. BUFFERS

6.1 PHAGE BUFFER

6.1.1 T2 Buffer

Solution A.	$KH_2\ PO_4$	7,5 g
	NaCl	20,0 g
	Anhydrous Na_2HPO_4	15,0 g
	K_2SO_4	25,0 g
	$CaCl_2$	0,055 g
	Dist H_2O	1000 ml

Stored over 10% chloroform.

Solution B.	$MgSO_4$	4,8 g
	Dist. H_2O	100,0 ml
	Autoclaved.	
Solution C.	Gelatin	0,2 g
	Dist. H_2O	100,0 ml
	Autoclaved.	
To make up:	Soln A	40,0 ml
	dist. H_2O	158,0 ml
	Soln B	1,0 ml
	Soln C	1,0 ml
	Autoclaved.	

6.2 GENERAL BUFFERS

6.2.1 Phosphate Buffer 0,2 M

A. 0,2 M soln of $NaH_2PO_4 .2H_2O$

B. 0,2 M soln of $Na_2H PO_4$

pH	A	B
5,8	46,0	4,0
6,0	43,85	6,15
6,2	40,75	9,25
6,4	36,75	13,25
6,6	31,25	18,75
6,8	25,5	24,5
7,0	19,5	30,5
7,2	14,0	36,0
7,4	9,5	40,5
7,6	6,5	43,5
7,8	4,25	45,75
8,0	2,65	47,35

6.2.2 Tris Buffers

- i) 0,02 M Tris HCl buffer, pH 8 adjusted with HCl.
- ii) 0,1 M Tris HCl buffer, pH 7,6 adjusted with HCl.
- iii) 0,1 M Tris maleate buffers, pH 6,0 and 9,0.

  12,1 g Tris + 11,6 g maleic acid or 9,8 g maleic anhydride in 1000ml distilled H_2O, adjusted to pH 6,0 and 9,0 with NaOH.

6.2.3 3 M Acetate Buffer

3 N acetic acid	100 ml
3 N NaOH	43 ml

pH adjusted to 4,5.

7. HOLDING SOLUTIONS

7.1 AEROBIC HOLDING SOLUTIONS

7.1.1 Ringer Solution (RS)

NaCl	2,25 g
KCl	0,105 g
$CaCl_2$	0,12 g
$NaHCO_3$	0,05 g
Dist. H_2O	1,0 l

7.1.2 Anaerobe Solution

Mineral Solution I

K_2HPO_4	0,6 g
Dist. H_2O	100,0 ml

Stored over chloroform in fridge.

Mineral Solution II

NaCl	1,2 g
$(NH_4)_2SO_4$	1,2 g
KH_2PO_4	0,6 g
$CaCl_2$ (anhydrous)	0,12 g
$MgSO_4.7H_2O$	0,25 g
Dist. H_2O	100,0 ml

Constituents dissolved one at a time in the above order. Stored over chloroform in fridge.

To make up

Mineral solution I	7,5 ml
Mineral solution II	7,5 ml
H_2O	100,0 ml

dispensed aerobically in Hungate tubes in 9 ml volumes. Autoclaved.

7.1.3 Minimal Salt Solution

Mineral soln	5 ml
$FeSO_4.7H_2O$	1 ml
$(NH_4)_2SO_4$	5 ml
Dist. H_2O	90 ml

dispensed aerobically in Hungate tubes in 9 ml volumes. Autoclaved.

7.1.4 BHl Solution

BHl	3,7 g
Yeast extract	0,5 g
Dist. H_2O	100 ml

Steamed for 30 min to dissolve, dispensed aerobically in Hungate tubes in 9 ml volumes and autoclaved.

7.2 ANAEROBIC HOLDING SOLUTIONS

7.2.1 Ringer Solution (RS)

Ringer solution	100,0 ml
Sodium thioglycollate	0,11 g
Na_2CO_3	0,4 g
Resazurin stock soln.	1,0 ml

Steamed for 30 min to reduce. Dispensed into 9 ml volumes, gassed and autoclaved.

7.2.2 Anaerobe Solution (AS)

Mineral solution I	7,5 ml
Mineral solution II	7,5 ml
Sodium thioglycollate	0,11 g
Na_2CO_3	0,4 g
Dist. H_2O	85,0 ml
Resazurin stock soln.	1,0 ml

Steamed for 30 min to reduce, dispensed into Hungate tubes in 9 ml volumes, gassed and autoclaved.

7.2.3 Minimal Salt Solution (MS)

Mineral salts	5,0 ml
$FeSO_4 . 7H_2O$	1,0 ml
$(NH_4)_2 SO_4$	5,0 ml
Dist. H_2O	90,0 ml
Sodium thioglycollate	0,11 g
Na_2CO_3	0,4 g
Resazurin stock soln.	1,0 ml

Steamed for 30 min to reduce. Dispensed into 9 ml volumes, gassed and autoclaved.

7.3 HOLDING SOLUTIONS WITH ADDITIVES

7.3.1 Inhibitors

Caffeine, sodium arsenite and acriflavine were added to liquid holding solutions at final concentrations of, caffeine:- 2,5; 5,0 and 50 mg ml^{-1}, sodium arsenite:- 100 μg ml^{-1}, and acriflavine:- 0,25; 1,0 and 2,5 μg ml^{-1}.

7.3.2 Antibiotics

Chloramphenicol and nalidicic acid were added to liquid holding solutions at final concentrations of, chloramphenicol:- 1 and 10 μg ml^{-1} and nalidicic acid:- 150, 300 and 600 μg ml^{-1}.

7.3.3 Nutrients

Yeast extract, peptone and peptone with tryptone were added to liquid holding solutions at final concentrations of yeast extract:- 0,75 mg ml^{-1}, peptone:- 15 mg ml^{-1} and 15 mg ml^{-1} peptone with 15 mg ml^{-1} of tryptone (30 mg ml^{-1}).

APPENDIX III

THE FORMATION OF SIMPLE FRUITING BODY-LIKE STRUCTURES ASSOCIATED WITH SPORULATION UNDER AEROBIC CONDITIONS IN *CLOSTRIDIUM ACETOBUTYLICUM*

The following work which forms part of a general program on anaerobic bacteria, was undertaken during the project on the *Bacteriodes fragilis* group, and has been accepted for publication (Journal of General Microbiology, 1980)

INTRODUCTION

The only group of bacteria known to produce differentiated fruiting bodies are the myxobacteria (Dworkin, 1966, 1973). Fruiting bodies produced by the myxobacteria vary from simple mounds of cells formed by *Myxococcus* to elaborate lobed forms found in the higher myxobacteria (*Polyangiaceae*). Within the fruiting body the individual vegetative cells lose their gliding motility and become resting cells (myxospores) which in some species are contained within cysts and in others occur as an undifferentiated mass. Although a number of other bacteria do show rudimentary multicellular organization (e.g. *Actinomycetes* (Chater & Hopwood, 1973)) no other group is known to produce fruiting body-like structures.

During a study of bacteriocin production by *Clostridium acetobutylicum* (Barber *et al.*, 1979), we observed the formation of elongated macroscopic structures resembling fruiting bodies. We describe here the production and nature of these structures.

METHODS

The *Clostridium acetobutylicum* strain was supplied by National Chemical Products Ltd., Germiston, South Africa. It was grown on the potato medium of Barber *et al.* (1979). Samples (0,5 g) of soil spore cultures of *C. acetobutylicum* was first heat-shocked in 3 ml 0,85% (w/v) NaCl at 70°C for 2 min before being inoculated into potato broth. Cultures were incubated at 34°C for 18 h and plated on potato agar. Duplicate plates were incubated in BBL GasPak jars for a further 48 h. One set of plates was then removed and incubation was continued aerobically at 20°C and the other set was retained under anaerobic conditions at 20°C. Colonies from each set of plates were examined macroscopically and microscopically every 2 d for 28 d. Spore and viability counts were carried out on colonies from both sets of plates. Wet-mounted specimens were observed with a Zeiss photomicroscope fitted with phase contrast optics. Metal-shadowed specimens were examined with a Jeol JSN U3 scanning electron microscope. Photographs of the macroscopic structures were taken with a Pentax SP 500 camera fitted with close-up rings.

RESULTS

Aerobic fruiting body-like structures

Plates containing isolated colonies of *C. acetobutylicum* were grown under anaerobic conditions for 2 d and then incubated aerobically for up to 4 weeks. Under aerobic conditions the colonies developed into unique elongated macroscopic structures which reached a height of > 10 mm over 3 to 4 weeks (Fig. 1a, b). These structures only developed in isolated colonies under aerobic conditions and crowding inhibited their development. The final shape of the macroscopic structures depended on the size of the colony at the time of transfer to aerobic conditions. Small colonies produced tall slender structures with a distinct helical twist (Fig. 1a) and large colonies produced shorter thicker structures.

Dissection and microscopic examination of the elongated structures indicated that they were associated with sporulation. Healthy rod-shaped vegetative cells and sporulating cells were restricted to the basal region of the structure (Fig. 1e). The trunk region contained mainly free spores (refractile bodies) and no sporulating cells were observed (Fig. 1f). There were only a few cells in the trunk region and they were either granulated or lysed in appearance.

Vegetative rods and spores within the macroscopic structure were surrounded by an extracellular substance which formed a tough pliable sheath around the colony. The difference between the surface layer (sheath) and the contents of the elongated colony was clearly seen under the scanning electron microscope in colonies which had been fractured during preparation (Fig. 1c, d). Closely packed vegetative rods near the surface appeared to be covered by a fibrous sheath (Figs. 1d and 2a).

The difference between the surface of an elongated structure and the surface of an anaerobic colony is shown in Fig. 2 (a,b). In the anaerobic colony the individual rod-shaped cells were clearly visible, whereas in the elongated structure the cells were covered by the fibrous sheath. Material from the sheath was dispersed in aqueous solution and observed microscopically. Most of the individual rods appeared to have lysed and were surrounded by an extracellular layer of refractile material (Fig. 1g).

It was possible to scrape out the soft inner contents of the elongated structure so that only the tough pliable outer sheath remained. After the removal of the inner contents the sheath became hard and brittle. When the surface of the elongated structure was damaged by cutting away the sheath and exposing the inner contents to air, a new tough pliable sheath was rapidly reformed over the damaged area.

Preliminary studies on the chemical composition of the sheath material indicated that it was proteinaceous. It was degraded by the proteolytic enzyme Pronase (Miles-Seravac) but unaffected by cellulysin (Calbiochem) or lysozyme (Miles-Seravac). It was soluble in sodium dodecyl sulphate but insoluble in water, hot alcohol, chloroform, ether, acetone, butanol, 2M-HCl or 5M-NaOH.

A solution of sheath material in sodium dodecyl sulphate absorbed ultraviolet light (280 nm) and could be assayed by the Folin-Ciocalteau test for proteins.

Aerobic development of fruiting body-like structures

The development of the elongated structures was related to the amount of sporulation which occurred within the colony. Sporulation in *C. acetobutylicum* grown on potato agar, under both aerobic and anaerobic conditions, was dependent on the crowding of the colonies on the surface of the plates. The closer the colonies were together, the lower the proportion of cells which sporulated. Crowded colonies did not produce the elongated sporulating structures.

After 48 h growth under anaerobic conditions, isolated colonies were greyish white, very flat with a diffuse margin (Fig. 2c), and contained only vegetative rods. After 48 h the cells within the isolated colonies began to sporulate under both anaerobic and aerobic conditions. The area of sporulation developed from the centre of aerobic colonies and was easily visible as a thicker and more opaque zone (Fig. 2c). This zone gradually spread outwards until after 3 to 4 d the entire colony was uniformly thick and opaque. The colony continued to develop vertically and over 14 d the proportion of rods which were healthy in appearance remained constant at about 30% of the total cell population. Sporulating cells accounted for about 60% of the cells. After 14 d the number of rods and sporulating cells decreased, with a concomitant increase in the number of free spores (about 30%) and granulated or lysed rods (about 60%).

In crowded colonies little growth occurred after 48 h. The colonies did not increase in size and remained thin and greyish white. After 6 d about 80% of the cells were granulated or lysed rods, the remainder being vegetative rods and sporulating cells. Only a few free spores were present after 10 d and after 21 d about 10% of the cells were free spores.

Anaerobic colony development

Under anaerobic conditions, isolated colonies produced typical flat spreading colonies which continued to increase in diameter over a period of 28 d. After 14 d, when the colonies were about 5 mm in diameter, large spreading fans grew out from the margins (Fig. 2d, f); these consisted entirely of vegetative rods (Fig. 2e) which contrasted with the actively sporulating cells making up the rest of the colony (Fig. 2e).

DISCUSSION

Under aerobic conditions isolated colonies of the anaerobe *C. acetobutylicum* produced an elongated structure which contained a high proportion of sporulating cells and free spores enclosed in a tough pliable sheath. This structure seemed to be intermediate between the ordinary bacterial colony and the elaborate multicellular fruiting bodies formed by myxobacteria, and could be regarded as an example of primitive multicellular differentiation.

We have recently examined two other strains of *C. acetobutylicum* (ATCC 824 and ATCC 10132) but have not as yet observed the production of fruiting body-like structures. However, these strains do not grow as well as our strain on potato agar.

The production under aerobic conditions of an extracellular proteinaceous substance which rapidly hardens on exposure to air to form a sheath is interesting. An obvious suggestion is that it acts as a protective barrier against oxygen. *Clostridium acetobutylicum* and most other clostridia are fairly strict anaerobes. Free oxygen inhibits growth but *C. acetobutylicum* can grow in liquid culture in the presence of air provided a sufficiently low oxidation reduction potential is established in the medium (Morris & O'Brien, 1971; O'Brien & Morris, 1971). *Clostridium acetobutylicum* will not normally grow on solid media under aerobic conditions. *Clostridium histolyticum, C. tertium* and *C. carnis* are exceptional in being able to grow aerobically to a limited extent (Wilson & Miles, 1975). Small but visible colonies are formed on blood agar plates but no spores are produced.

Under anaerobic conditions on potato agar, *C. acetobutylicum* produced flat spreading colonies with fans of vegetative cells. This contrasts with the report of Hastings (1978) who observed that on a molasses agar (under anaerobic conditions) colonies darken slightly and grow in the form of a truncated cone, 2 to 3 mm high, with a concave top. We have only observed the production of the elongated structures, which are not truncated and do not have a concave top, under aerobic conditions.

FIG. 1: Scanning electron micrographs (a to d) and photomicrographs (e to g) showing the morphology and structure of elongated fruiting body-like structures produced by *C. acetobutylicum*. (a) Elongated structure with a helical twist; (b) development of elongated structures over a 4 week period (right to left); (c,d) fractured elongated structures showing the tough pliable sheath; (e) vegetative rods and sporulating cells from the basal region of the structure; (f) trunk region showing free spores (refractile bodies) and a few cells which were either granulated or lysed in appearance; (g) sheath material dispersed in an aqueous solution showing lysed rods surrounded by extracellular refractile material.

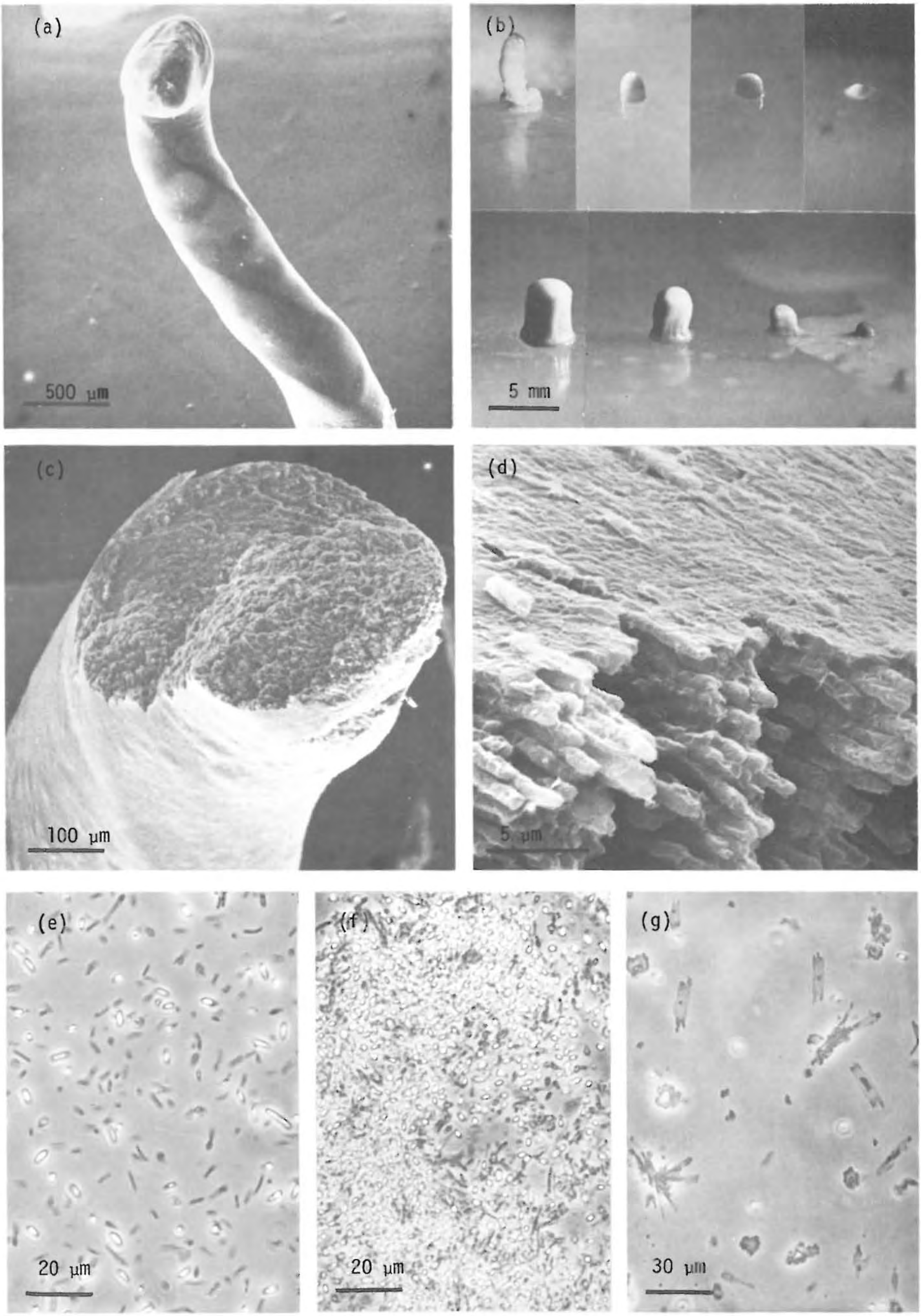
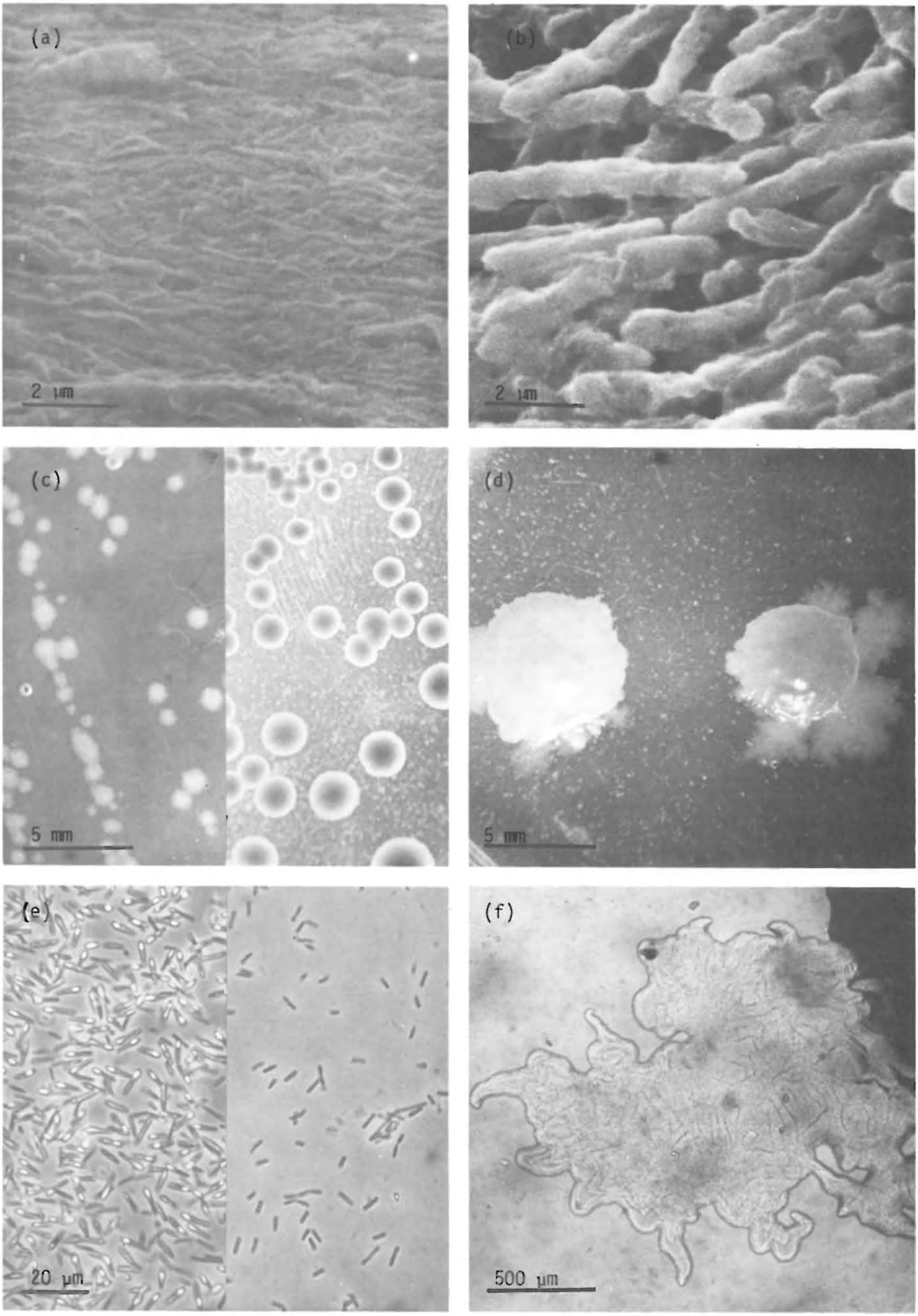

(a)
500 µm
(b)
5 mm
(c)
100 µm
(d)
5 µm
(e)
20 µm
(f)
20 µm
(g)
30 µm

FIG. 2: Scanning electron micrographs (a,b) and photomicrographs (c to f) showing the morphology and structure of aerobic and anaerobic colonies of *C. acetobutylicum*. (a) Surface of an aerobic elongated structure; (b) surface of an anaerobic colony; (c) greyish white flat colonies after 48 h growth under anaerobic conditions (left) and the development of the thicker central sporulating zone after 72 h (right); (d) anaerobic colonies showing the spreading fan of vegetative cells; (e) sporulating cells from the centre of anaerobic colonies (left) and vegetative rods from the fan region of an anaerobic colony (right); (f) spreading fan of an anaerobic colony.


(a)
2 µm
(b)
2 µm
(c)
5 mm
(d)
5 mm
(e)
20 µm
(f)
500 µm

REFERENCES

Achey, P. & Billen, D. 1969. Saturation of dark repair synthesis: Accumulation of strand breaks. *Biophys.J.* 9:647.

Adams, M.H. 1959. *The Bacteriophages.* New York: Interscience Publishers.

Adelberg, E.A., Mandel, M. & Chen, G.C.C. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K12. *Biochem.Biophys.Res.Commun.* 18:788.

Alder, H.I. & Hardigree, A.A. 1965. Postirradiation growth, division and recovery in bacteria. *Radiat.Res.* 25:92.

Alper, T. 1963. Effects on irradiated micro-organisms of growth in the presence of acriflavine. *Nature* 200:534.

Alper, T. & Gillies, N.E. 1958. "Restoration" of *E.coli* strain B after irradiation: Its dependence on suboptimal growth conditions. *J.Gen.Microbiol.* 18:461.

Alper, T. & Gillies, N.E. 1960. The relationship between growth and survival after irradiation of *E.coli* strain B and two resistant mutants. *J.Gen.Microbiol.* 22:113.

Anderson, J.A. & Barbour, S.D. 1973. Effects of thymine starvation on deoxyribonucleic acid repair systems of *E.coli* K-12. *J.Bacteriol.* 113:114.

Anderson, J.D. & Sykes, R.B. 1973. Characterization of a β-lactamase obtained from a strain of *Bacteroides fragilis* resistant to β-lactamase antibiotics. *J.Med.Microbiol.* 6:201.

Anderson, P.S. & Cowles, P.B. 1958. Effect of antiphage serum on the virulence of *Corynebacterium diphtheriae.* *J.Bacteriol.* 76:272.

Aranki, A., Syed, S.A., Kenney, E.B. & Freter, R. 1969. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. *Appl.Microbiol.* 17:568.

Arditti, R.R. & Coppo, A. 1965. Effect of acridines and temperature on a strain of *Bacillus megaterium* lysogenic for phage α. *Virology* 25:643.

Askevold, F. 1956. Investigations on the influence of diet on the quantity and composition of intestinal gas in humans. *Scand.J.of Clin.Lab.Invest.* 8:87.

Babb, J.L. & Cummins, C.S. 1978. Encapsulation of *Bacteroides* Species. *Infect.Immun.* 19:1088.

Bachmann, B.J., Low, K.B. & Taylor, A.L. 1976. Recalibrated linkage map of *E.coli* K-12. *Bacteriol.Rev.* 40:116.

Baess, I. 1971. Report on a pseudolysogenic *Mycobacterium* and a review of the literature concerning pseudolysogeny. *Acta Path.Microbiol. Scand.* 79:428.

Bailone, A., Levine, A. & Devoret, R. 1979. Inactivation of prophage λ repressor *in vivo*. *J.Bol.Biol.* 131:553.

Baker, R., Doniger, J. & Tessman, I. 1971. Roles of parental and progeny DNA in two mechanisms of phage S13 recombination. *Nature.New.Biol.* 230:23

Barber, J.M., Robb, F.T. Webster, J.R. & Woods, D.R. 1979. Bacteriocin production by *Clostridium acetobutylicum* in an industrial fermentation process. *Appl.Environ.Microbiol.* 37:433.

Barner, H.D. & Cohen, S.S. 1956. The relation of growth to the lethal damage induced by ultraviolet irradiation in *E.coli*. *J.Bacteriol.* 71:149.

Baron, L.S., Penido, E., Ryman, I.R. & Falkow, S. 1970. Behaviour of coliphage lambda in hybrids between *Escherichia coli* and *Salmonella*. *J.Bacteriol.* 102:221.

Bartlett, J.G. & Onderdonk, A.B. 1979. Virulence factors of anaerobic Bacteria: Summary of the Symposium. *Rev.Infect.Dis.* 1:398.

Beerens, H., Schaffner, Y., Guillaume, J. & Castel, M.M. 1963. Les bacilles anaerobies non sporules a gram negatif favorises par la bile. Leur appartenance au genre *Eggerthella*. *Ann.Inst.Pasteur* (Lille) 14:5.

Beerens, H., & Baron, G. 1965. Mise en évidence de bactéroicines élaborées par les bactéries anaérobies a gram negatief appartenant au genre *Eggerthella*. *Ann.Inst.Pasteur* (Paris) 108:255.

Beerens, H., Baron, G. & Tahon, M.M. 1966. Mise en évidence de substraces inhibitres élaborées par les bacteries anaérobies non sporulees. *Ann.Inst.Pasteur* (Lille) 17:1.

Benzer, S. & Freese, E. 1958. Induction of specific mutations with 5- Bromouracil. *Proc.Nat.Acad.Sci.* 44:112.

Bernheimer, H.P. & Tiraby, J. 1976. Inhibition of phage infection by *Pneumococcus* capsule. *Virology* 73:308.

Bernstein, C., Morgan, D., Gensler, H., Schneider, S. & Holmes, G. 1976. The dependence of HNO_2 mutagenesis in T4 on ligase and the lack of dependence of 2 AP mutagenesis on repair functions. *Mol.Gen.Genet.* 148:213.

Beukers, R. & Berends, W. 1960. Isolation and identification of the irradiation product of thymine. *Biochim.Biophys.Acta* 41:550.

Billen, D. 1969. Replication of the bacterial chromosome location of new initiation sites after irradiation. *J.Bacteroil.* 97:1169.

Billen, D., Hewitt, B., Lapthisophon, A., & Achey, P. 1967. Deoxyribonucleic acid repair replication after ultraviolet light or x-ray exposure of bacteria. *J.Bacteriol.* 94:1538.

Billen, D. & Bruns, L.H. 1970a. Dampening of DNA replication cycles after X-irradiation or UV-irradiation. *Biophys.J.* 10:509.

Billen, D. & Bruns, L.H. 1970b. Relationship between radiation response and the deoxyribonucleic acid replication cycle in bacteria: Dependence on the excision-repair system. *J.Bacteriol.* 103:400.

Billen, D. & Carreira, L.B. 1971. Characterization of DNA synthesis in an *Hcr* mutant of *Escherichia coli* exposed to ultraviolet light. *Microbiol.* 3:153.

Blanco, M. & Devoret, R. 1973. Repair mechanisms involved in prophage reactivation and UV reactivation of UV-irradiated phage λ. *Mutat.Res.* 17:293.

Bonura, T. & Smith, K.C. 1975. Enzymatic production of deoxyribonucleic acid double strand breaks after UV irradiation of *E.coli* K-12. *J.Bacteriol.* 121:511.

Booth, S.J., Johnson, J.L. & Wilkins, T.D. 1977. Bacteriocin production by strains of *Bacteroides* isolated from human feces and the role of these strains in the bacterial ecology of the colon. *Antimicro. Agents Chemother.* 11:718.

Booth, S.J., van Tassell, R.L., Johnson, J.L. & Wilkins, T.D. 1979. Bacteriophages of *Bacteroides*. *Rev.Infect.Dis.* 1:325.

Borek, E. & Ryan, A. 1958. The transfer of irradiation-elicited induction in a lysogenic organism. *Proc.Natl.Acad.Sci.USA* 44:374.

Bott, K. & Strauss, B. 1965. The carrier state of *Bacillus subtilis* infected with the transducing bacteriophage SP10. *Virology* 25:212.

Boyce, R.P. & Howard-Flanders, P. 1964. Release of UV induced thymine dimers from DNA in *E.coli* K12. *Proc.Natl.Acad.Sci.USA.* 51:293.

Boyle, J.M. & Symonds, N. 1969. Radiation-sensitive mutants of T_4D.I. T_4y: A new radiation-sensitive mutant; effect of the mutation on radiation survival, growth and recombination. *Mutat.Res.* 8:431.

Boyle, J.M. & Setlow, R.B. 1970. Correlations between host-cell reactivation and pyrimidine dimer excision in the DNA of bacteriophage λ. *J.Mol.Biol.* 51:131.

Boyle, J.M. & Swenson, P.A. 1971. Loss of the capacity of UV-irradiated *E.coli* B/r to grow T4D. *Virology* 44:37.

Branche, W.C. Jr., Young, V.M., Robinet, H.G. & Massey, E.M. 1964. Effect of colicine production of *Escherichia coli* in the normal human intestine. *Soc.Exp.Biol.Med.* 114:148.

Brandis, H., Voight, W.H. & Viebahn, A. 1972. Morphological and biological properties of the *Bacteroides fragilis* bacteriophage ØAI. *Zbl.Bakt.Hyg.,* I.Abt. Orig.A 222:57.

Braun, A. & Grossman, L. 1974. An endonuclease from *Escherichia coli* that acts preferentially in UV-irradiated DNA and is absent from the *uvr*A and *uvr*B mutants. *Proc.Natl.Acad.Sci.USA.* 71:1838.

Braun, A., Hopper, P. & Grossman, L. 1975. The *Escherichia coli* UV endonuclease (Correndonuclease II). In *Molecular Mechanisms for Repair of DNA* (eds P.C. Hanawalt & R.B. Setlow), p.183. New York: Plenum Press.

Brenner, D., Gilchrist, J.E. & De Voe, I.W. 1977. Relationship between colonial variation and pili morphology in a strain of *Neisseria meningitidis*. *F.E.M.S. Micro Letters* 2:157.

Bridges, B.A. 1972. Evidence for a further dark repair process in bacteria. *Nat.New.Biol.* 240:52.

Bridges, R.A. 1978. The involvement of *E.coli* DNA Polymerase III in constitutive and inducible mutagenic repair. In *DNA repair mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C. Fox), New York, San Fransisco, London: Academic Press.

Bridges, B.A. & Munson, R.J. 1968. The presistence through several replication cycles of mutation-producing pyrimidine dimers in a strain of *E.coli* deficient in excision repair. *Biochem.Biophys. Res.Comm.* 30:620.

Bridges, B.A., Mottershead, R.P. & Sedgwick, S.G. 1976. Mutagenic DNA repair in *E.coli*. *Mol.Gen.Genet.* 144:53.

Brown, D. & Gillies, N.E. 1972. The relationship between filaments, killing and restoration in irradiated *E.coli* strain B. *J.Gen.Microbiol.* 70:461.

Brunschede, H. & Bremer, H. 1969. Protein synthesis in *E.coli* after irradiation with UV light. *J.Mol.Biol.* 41:25.

Bryant, M.P. & Burkey, L.A. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J.Dairy Science* 36:205.

Bukhari, A.I. 1976. Bacteriophage Mu as a transposition element. *Ann.Rev.Genetics* 10:389.

Bukhari, A.I., Shapiro, J.A. & Adhya, S.L. 1977. DNA insertion elements, plasmids and episomes. Cold Spring Harbour Laboratory.

Burman, L.G. 1975. Amplification of sex repressor function of one fi^+ R-factor during anaerobic growth of *Escherichia coli*. *J.Bacteriol.* 123:265.

Burt, S.J. 1977. Genetic and bacteriophage studies on *Bacteroides thetaiotaomicron* and related anaerobic strains. Ph.D. Thesis, Rhodes University, South Africa.

Burt, S.J. & Woods, D.R. 1976. R factor transfer to obligate anaerobes from *E.coli*. *J.Gen.Microbiol.* 93:405.

Burt, S.J. & Woods, D.R. 1977. Transfection of the anaerobe *Bacteroides thetaiotaomicron* with phage DNA. *J.Gen.Microbiol.* 103:181.

Burt, S.J., Meldrum, S., Woods, D.R. & Jones, D.T. 1978. Colonial variation, capsule formation and bacteriophage resistance in *Bacteroides thetaiotaomicron*. *Appl.Environ.Microbiol.* 35:439.

Campbell, A.M. 1962. Episomes. *Adv.Genetics* 11:109.

Caldwell, D.R. & Arcand, C.A. 1974. Inorganic and metal-organic growth requirements for genus *Bacteroides*. *J.Bacteriol.* 120:322.

Carlson, J., Wrethen, J. & Beckman, G. 1977. Superoxide dismutase in *Bacteroides fragilis* and related *Bacteroides* species. *J.Clin. Microbiol.* 6:280.

Carrier, W.L. & Setlow, R.B. 1971. The excision of pyrimidine dimers. *Meth. in Enzy.* 21:230.

Castellani, A., Jagger, J. & Setlow, R.B. 1964. Overlap of photoreactivation and liquid holding recovery in *E.coli* B. *Science* 143:1170.

Cato, E.P. & Johnson, J.L. 1976. Reinstatement of species rank of *Bacteroides fragilis, B.ovatus, B.distasonis, B.thetaiotaomicron* and *B.vulgatus:* Designation of neotype strains for *B.fragilis* (Veillon & Zuber) Castellani & Chalmers and *B.thetaiotaomicron* (Distaso) Castellani & Chalmers. *Int.J.Syst.Bacteriol.* 26:230.

Chakrabarty, A.M. & Gunsalus, I.C. 1969. Autonomous replication of a defective transducing phage in *Pseudomonas putida*. *Virology* 38:92.

Chase, M.C. 1964. Ph.D. Thesis, University of Southern California, Los Angeles, California.

Chater, K.F. & Hopwood, D.A. 1973. Differentiation in *actinomycetes*. *Symp.Soc.Gen.Microbiol.* 23:143.

Chow, A.W. & Guze, L.B. 1974. *Bacteroidaceae* bacteremia: Clinical experience with 112 patients. *Medicine* 53:93.

Clark, A.J. & Ganesan, A. 1975. Lists of genes affecting DNA metabolism in *E.coli*. In *Molecular Mechanisms for repair of DNA* (eds P.C. Hanawalt & R.B. Setlow), p.431. New York: Plenum Press.

Clarke, N.A. 1952. Studies on the host-virus relationship in a lysogenic strain of *Bacillus megaterium*. II. The growth of *B.megaterium* in synthetic medium. *J.Bacteriol.* 63:187.

Cohen, S.S. 1971. On the nature of thymineless death. *Ann.NY.Acad.Sci.* 186:292.

Cohen, S.S. & Arbogast, R. 1950. The mutual reactivation of T2r$^+$ virus inactivated by ultraviolet light and the synthesis of desoxyribase nucleic acid. *J.Exp.Med.* 91:637.

Cook, J.S. 1970. Photoreactivation and photoreactivating enzymes. *Photophysiology* 5:191.

Cooke, E.M., Hettiaratchy, I.G.T. & Buck, A.C. 1972. Fate of ingested *Escherichia coli* in normal persons. *J.Med.Microbiol.* 5:361.

Cooper, P.K. & Hannawalt, P.C. 1972. Heterogenicity of patch size in repair replicated DNA in *E.coli*. *J.Mol.Biol.* 67:1.

Cunningham, R.P., Shibata, T., Das Gupta, C. & Radding, C.M. 1979. Single strands induce *rec* A protein to unwind duplex DNA for homologous pairing. *Nature* 281:191.

Damle, S.P. & Syed, S.A. 1975. Demonstration of plasmid DNA in *Bacteroides* species of oral origin. *J.Dent.Res.* 54:104.

Deering, R.A. 1958. Studies on division inhibition and filament formation of *E.coli* by UV light. *J.Bacteroil.* 76:123.

Deering, R.A. & Setlow, R.B. 1957. Inhibition of cell division of *E.coli* by low doses of untraviolet light. *Science* 126:397.

Defais, M., Fauquet, P., Radman, M. & Errera, M. 1971. Ultraviolet reactivation and ultraviolet mutagenesis of λ in different genetic systems. *Virology* 43:495.

Del Bene, V.E., Rogers, M. & Farrar, W.E. 1976. Attempted transfer of antibiotic resistance between *Bacteroides* and *Escherichia coli.* *J.Gen.Microbiol.* 92:384.

Devoret, R. & Coquerell, T. 1966. Genetic aspects of radiosensitivity: mechanisms of repair. p.89. Vienna: International Atomic Energy Agency.

Devoret, R., Blanco, M., George, J. & Radman, M. 1975. Recovery of phage λ from ultraviolet damage. In *Molecular mechanisms for repair of DNA* (eds P.C. Hanawalt & R.B. Setlow). New York: Plenum Press.

Doermann, A.H. 1961. The analysis of UV lesions in bacteriophage T4 by cross reactivation. *J.Cell.Comp.Physiol.* Suppl. 1:79.

Domon, M., Barton, B., Porte, A. & Rauth, A.M. 1970. The interaction of caffeine with ultraviolet-light-irradiated DNA. *Int.J.Radiat.Biol.* 17:395.

Donch, J. & Greenberg, J. 1968. Ultraviolet sensitivity gene of *E.coli* B. *J.Bacteriol.* 95:1555.

Donch, J.J., Green, M.H.L. & Greenberg, J. 1968. Interaction of the *exr* + *lon* genes in *E.coli.* *J.Bacteriol.* 96:1704.

Donch, J., Chung, Y.S. & Greenberg, J. 1969. Locus for radiation resistance in *E.coli* strains B/r. *Genetics* 61:363.

Doudney, C.O. 1959. Macromolecular synthesis in bacterial recovery from UV light. *Nature* 184:189.

Doudney, C.O. 1965. Ultraviolet light effects on DNA replication. In *Cellular Radiation Biology,* p.120. Baltimore: Williams & Wilkins.

Doudney, C.O. 1966. Photoreversal of ultraviolet light induced mutation, lethality and damage to nucleic acid formation in bacteria. *Mutat.Res.* 3:280.

Doudney, C.O. 1968. UV light effects on the bacterial cell. *Curr. Top.Microbiol.Immunol.* 46:116.

Doudney, C.O. 1971. Increased resistance of DNA replication to ultraviolet light damage in amino acid-starved bacteria. *Biochim.Biophys.Acta* 228:57.

Drakulič, M. & Errera, M. 1959. Cloramphenicol-sensitive DNA synthesis in normal and irradiated bacteria. *Biochim.Biophys.Acta* 31:459.

Drakulič, M., Kos, E.& Brdar, B. 1966. Comparison of survival of a UV-irradiated uracil-requiring strain of *E.coli* B in the presence of chloramphenicol and in the absence of the essential metabolite. *Photochem.Photobiol.* 5:265.

Drasar, B.S. 1967. Cultivation of anaerobic intestinal bacteria. *J.Path.Bacteriol.* 94:417.

Drasar, B.S. & Hill, M.J. 1974. *Human Intestinal Flora.* London: Academic Press.

Drozd, J. & Postgate, J.R. 1970. Effects of oxygen on acetylene reduction, cytochrome content and respiratory activity of *Azotobacter chroococcum.* *J.Gen.Microbiol.* 63:63.

Dubos, R., Schaedler, R.W. & Costello, R. 1963. Composition, alteration and effects of the intestinal flora. *Fed.Proc.* 22:1322.

Duguid, J.P. & Gillies, R.R. 1958. Fimbriae and haemagglutinating activity in *Salmonella, Klebsiella, Proteus* and *Chromobacterium.* *J.Path.Bact.* 75:519.

Duguid, J.P. & Wilkinson, J.K. 1961. Environmentally induced changes in bacterial morphology. *Symp.Soc.Gen.Microbiol.* 11:69.

Dulbecco, R. 1950. Experiments on photoreactivation of bacteriophages inactivated with UV radiation. *J.Bacteriol.* 59:329.

Dulbecco, R. 1952. A critical test of the recombination theory of multiplicity reactivation. *J.Bacteriol.* 63:199.

Durham, N.M. & Wyss, O. 1956. An example of non-inherited radiation resistance. *J.Bacteriol.* 72:95.

Dworkin, M. 1966. Biology of the *myxobacteria.* *Ann.Rev.Microbiol.* 20:75.

Dworkin, M. 1973. Cell - cell interactions in the *myxobacteria.* *Symp.Soc.Gen.Microbiol.* 23:125.

Eggerth, A.H. & Gagnon, B.H. 1933. The *Bacteroides* of human feces. *J.Bacteriol.* 25:389.

Eisenstark, A. 1977. Genetic recombination in bacteria. *Ann.Rev.genet.* 11:369.

Eklund, M.W., Poysky, F.T., Reed, S.M. & Smith, C.A. 1971. Bacteriophage and the toxigenicity of *Clostridium botulinium* type C. *Science* 172:480.

Elad, D. 1976. The role of nucleic acid additional reactions. In *Aging Carcinogenesis and Radiation Biology* (ed. K.C. Smith), p.243. New York: Plenum Press.

Emmerson, P.T. & West, S.C. 1978. Induction and repression of the *rec* A gene of *Escherichia coli*. In *DNA repair mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C.F. Fox). New York, San Fransisco, London: Academic Press.

Epstein, R.H. 1958. A study of multiplicity reactivation in the bacteriophage T4. Genetic and functional analysis of T4D - K12 (λ) complexes. *Virology* 6:382.

Errera, M. 1954. Induction of filamentous forms in ultraviolet-irradiated *E.coli* B. *Br.J.Radiol.* 27:76.

Falkow, S. & Baron, L.S. 1970. Plasmid formation after lambda bacteriophage infection of *Escherichia coli* - *Salmonella typhosa* hybrids. *J.Bacteriol.* 102:288.

Feiner, R.R. & Hill, R.F. 1963. Effect of basic dyes on host-cell reactivation of ultraviolet-irradiated phage. *Nature* 200:291.

Felner, J.M. & Dowell, V.R. 1971. *Bacteroides* bacteremia. *Am.J.Med.* 50:787.

Finegold, S.M. 1969. Intestinal bacteria - the role they play in normal physiology, pathologic physiology and infection. *Calif.Med.* 110:455.

Finegold, S.M. 1970. Gram-negative anaerobic rods - *Bacteroidaceae*. In *Gradwahl's Clinical Laboratory Methods and Diagnosis*, (eds S. Frankel *et al.*), 7th edition, p.1353. St.Louis: C.V. Mosby.

Finegold, S.M. & Sutter, V.L. 1971. Susceptibility of Gram-negative anaerobic bacilli to gentamycin and other aminoglycosides. *J.Infect.Dis.* 124:556.

Finegold, S.M., Shepard, W.E., & Spaulding, E.H. 1977. Practical anaerobic bacteriology. CUMITECH no. 5, *Am.Soc.Microbiol.*, Washington D.C. p.1.

Fisher, K.W. 1957. The role of the Krebs cycle in conjugation in *Escherichia coli* K12. *J.Gen.Microbiol.* 16:120.

Fisher, W.D., Adler, H.I., Shull, F.W. & Cohen, A. 1969. Properties of a cell fraction that repairs damage to the cell division mechanism of *E.coli*. *J.Bacteriol.* 97:500.

Fluke, D.J. & Pollard, E.C. 1949. Ultraviolet action spectrum of T1 bacteriophage. *Science* 110:274.

Fong, K. & Bockrath, R.C. 1979. Inhibition of DNA repair in *E.coli* by caffeine and acriflavine after UV irradiation. *J.Bact.* 139:671.

Forage, A.J. & Gillies, N.E. 1969. Enhancement of damage to *E.coli* strain B/r after ultraviolet and γ irradiation. *J.Gen.Microbiol.* 59:329.

Fredericq, P. 1957. Colicins. *Ann.Rev.Microbiol.* 11:7.

Fredericq, P. 1963. On the nature of colicinogenic factors: A review. *J.Theor.Biol.* 4:159.

Fredette, V., Plante, C. & Roy, A. 1967. Numerical data concerning the sensitivity of anaerobic bacteria to oxygen. *J.Bacteriol.* 94:2012.

Fridovich, I. 1972. Superocide radical and superocide dismutase. *Acc.Chem.Res.* 5:321.

Fridovich, I. 1975. Superoxide dismutases. *Ann.Rev.Biochem.* 44:147.

Fridovich, I. 1977. Oxygen is toxic! *Bio.Science* 27:462.

Friedberg, E.C. 1975. Dark Repair in bacteriophage systems: Overview. In *Molecular Mechanisms for Repair of DNA* (ed. P.C. Hanewalt & R.B. Setlow), p.125. New York: Plenum Press.

Friedberg, E.C. & King, J.J. 1969. Endonucleolytic cleavage of UV-irradiated DNA controlled by the V^+ gene in plage T4. *Biochem. Biophys.Res.Commun.* 37:646.

Friedberg, E.C. & King, J.J. 1971. Dark repair of UV-irradiated deoxyribonucleic acid by bacteriophage T4 purification and characterization of a dimer-specific phage-induced endonuclease. *J.Bacteriol.* 106:500.

Friedberg, E.C., Minton, K., Paul, G. & Verzola, P. 1974. Excision of thymine dimers *in vitro* by extracts of bacteriophage-infected *E.coli*. *J.Virol.* 13:953.

Friedberg, E.C., Cook, K.H., Duncan, J., & Mortelmans, K. 1977. In *Photochemical and Photobiological Reviews* (ed. K.C. Smith) Vol. 2, pp.263-322. New York: Plenum Press.

Friedberg, E.C., Bonura, T., Cone, R., Simmons, R. & Anderson, C. 1978. Base excision repair of DNA. In *DNA Repair Mechanisms* (ed. P.C. Hanawalt, E.C. Friedberg & C.F. Fox), pp.163-174. New York: Academic Press.

Ganesan, A.K. 1974. Persistence of pyrimidine dimers during post-replication repair in UV light irradiated *E.coli* K12. *J.Mol.Biol.* 87:103.

Ganesan, A.K. & Smith, K.C. 1968a. Dark recovery processes in *E.coli* irradiated with ultraviolet light. I. Effect of *rec*⁻ mutations on liquid holding recovery. *J.Bacteriol.* 96:365.

Ganesan, A.K. & Smith, K.C. 1968b. Recovery of recombination deficient mutants of *E.coli* K-12 from ultraviolet irradiation. *Cold Spring Harbor Symp.Quant.Biol.* 33:235.

Ganesan, A.K. & Smith, K.C. 1969. Dark recovery processes in *E.coli* irradiated with UV light. II. Effects of *uvr* genes on liquid holding recovery. *J.Bacteriol.* 97:1129.

Ganesan, A.K. & Smith, K.C. 1971. The duration of recovery and DNA repair in excision deficient derivatives of *Escherichia coli* K-12 after ultraviolet irradiation. *Mol.Gen.Genet.* 113:285.

Ganesan, A.K. & Smith, K.C. 1972. Requirement for protein synthesis in *rec*-dependent repair of deoxyribonucleic and in *E.coli* after UV or X irradiation. *J.Bacteriol.* 111:575.

Garen, A. & Zinder, N.D. 1955. Radiological evidence for partial genetic homology between bacteriophage and host bacteria. *Virology* 1:347.

George, J. & Devoret, R. 1971. Conjugal transfer of UV-damaged F-prime sex factors and indirect induction of prophage λ. *Mol.Gen.Genet.* 111:103.

George, J., Devoret, R. & Radman, M. 1974. Indirect ultraviolet reactivation of phage λ. *Proc.Natl.Acad.Sci.USA.* 71:144.

Gillies, N.E. 1961. The use of auxotrophic mutants to study restoration in *E.coli* B after ultraviolet irradiation. *Int.J.Radiat.Biol.* 3:379.

Ginsberg, D.M. & Jagger, J. 1965. Evidence that initial UV lethal damage in *E.coli* $15T^-A^-U^-$ is independent of growth phase. *J.Gen.Microbiol.* 40:171.

Grant, R.B. & Riemann, H.P. 1976. Temperate phages of *Clostridium perfringens*. *Canad.J.Microbiol.* 22:603.

Gregory, E.M., Yost, F.J. & Fridovich, I. 1973. Superoxide dismutases of *E.coli:* intracellular localization and functions. *J.Bacteriol.* 115:987.

Gregory, E.M. & Fridovitch, I. 1974. Oxygen metabolism in *Lactobacillus plantarum*. *J.Bacteriol.* 117:166.

Gregory, E.M., Kowalski, J.B. & Holdeman, L.V. 1977a. Production and some properties of catalase and superoxide dismutase from the anaerobe *Bacteroides distasonis*. *J.Bacteriol.* 129:1298.

Gregory, E.M., Veltri, B.J., Wagner, D.L. & Wilkins, T.D. 1977b. Carbohydrate repression of catalase synthesis in *Bacteroides fragilis*. *J.Bacteriol.* 129:534.

Gregory, E.M., Moore, W.E.C. & Holdeman, L.V. 1978. Superoxide dismutase in anaerobes: a survey. *Appl.Environ.Microbiol.* 35:988.

Green, M.H.L., Greenberg, J. & Donch, J. 1969. Effect of a *rec* A gene on cell division and capsular polysaccharide production in a *lon* strain of *E.coli*. *Genet.Res.Camb.* 14:159.

Grossman, L., Braun, A., Feldberg, R. & Mahler, 1975. I. Enzymatic repair of DNA. *Ann.Rev.Biochem.* 44:19.

Gudas, L.J. & Pardee, A.B. 1975. Model for regulation of *Escherichia coli* DNA repair functions. *Proc.Natl.Acad.Sci.USA.* 72:2330.

Guiney, D.G. & Davis, C.E. 1975. Isolation of plasmid deoxyribonucleic acid from two strains of *Bacteroides*. *J.Bacteriol.* 124:503.

Guiney, D.G. & Davis, C.E. 1978. Identification of a conjugative R plasmid in *Bacteroides ochraceus* capable of transfer to *E.coli*. *Nature* 274:181.

Haenel, H. 1970. Human normal and abnormal gastrointestinal flora. *Am.J.Clin,Nutr.* 23:1433.

Hager, L.P. 1973. *Chemistry and functions of colicins*. New York: Academic Press.

Hamkalo, B.A. & Swenson, P.A. 1969. Effects of ultraviolet radiation on respiration and growth in radiation-resistant and radiation-sensitive strains of *E.coli* B. *J.Bact.* 99:815.

Hanawalt, P.C. 1966. The UV sensitivity of bacteria: Its relation to the DNA replication cycle. *Photochem.Photobiol.* 5:1.

Hanawalt, P.C. & Setlow, R.B. 1960. Effect of monochromatic UV light on macromolecular synthesis in *E.coli*. *Biochim.Biophys.Acta* 41:283.

Hanawalt, P.C. & Haynes, R.H. 1967. The repair of DNA. *Scientific American* 216:36.

Hanawalt, P.C. & Setlow, R.B. *Molecular mechanisms for repair of DNA*. New York & London: Plenum Press.

Hanawalt, P.C., Friedberg, E.C. & Fox, C.F. *DNA repair mechanisms*. New York: Academic Press.

Hardy, K.G. 1975. Colicinogeny and related phenomena. *Bacteriol.Rev.* 39:464.

Hardy, K.G. 1978. Bacteriocins. In *Companion to Microbiology* (eds A.T. Bull & P.M. Meadow). London & New York: Longman.

Harm, W. 1961. Gene-controlled reactivation of ultraviolet-inactivated bacteriophage. *J.Cell.Comp.Physiol.* 48(Suppl. 1):69.

Harm, W. 1963a. On the relationship between host-cell reactivation and UV-reactivation in UV-inactivated phages. *Z.Vererbungsl.* 94:67.

Harm, W. 1963b. Mutants of phage T4 with increased sensitivity to ultraviolet. *Virology* 19:66.

Harm, W. 1966. The role of host-cell repair in liquid-holding recovery of UV-irradiated *E.coli*. *Photochem.Photobiol.* 5:747.

Harm, W. 1967. Differential effects of acriflavine and caffeine on various ultraviolet-irradiated *E.coli* strains and T1 phage. *Mutat.Res.* 4:93.

Harm, W. 1968a. Dark repair of photorepairable UV lesions in *E.coli*. *Mutat.Res.* 6:25.

Harm, W. 1968b. Effect of dose fraction on UV survival of *E.coli*. *Photochem.Photobiol.* 7:73.

Harm, W. 1970. Analysis of photoenzymatic repair of UV lesions in DNA by single light flashes. VIII. Inhibition of photoenzymatic repair of UV lesions in *E.coli* by caffeine. *Mutat.Res.* 10:319.

Harm, W. 1973. T4 mediated V-gene reactivation of UV-irradiated phage T1 and its comparison with host-cell reactivation. *Mutat.Res.* 17:163.

Harm, W. 1974. Dark recovery of UV irradiated T1. III. Evidence for two modes of host cell repair from liquid-holding experiments. *Mutat.Res.* 25:15.

Harm, W. 1975. Kinetics of photoreactivation. In *Molecular Mechanisms for repair of DNA* (Part A) (eds P.C. Hanawalt & R.B. Setlow). New York & London: Plenum Press.

Harm, W., Rupert, C.S. & Harm, H. 1971. *Photophysiology* Vol. 6 (ed. A.C. Giese), p.279. New York: Academic Press.

Harold, F.M. & Ziporin, Z.Z. 1958. Synthesis of protein and of DNA in *E.coli* irradiated with UV light. *Biochim.Biophys.Acta.* 29:439.

Hart, M.G.R., & Ellison, J. 1970. Ultraviolet reactivation in bacteriophage lambda. *J.Gen.Virol.* 8:197.

Hassan, H.M. & Fridovich, I. 1979. Superoxide, hydrogen peroxide, and oxygen tolerance of oxygen-sensitive mutants of *E.coli*. *Rev.Infect.Dis.* 1:357.

Hastings, J.J.H. 1978. Acetone-butyl alcohol fermentation. In *Economic microbiology* Vol. 2, (ed. A.H. Rose), p.31. New York & London: Academic Press.

Haynes, R.H. 1966. The interpretation of microbial inactivation and recovery phenomenon. *Radiat.Res.* 6:1.

Haynes, R.H., Baker, R.M. & Jones, G.E. 1968. Genetic implications of DNA repair. In *Energetics and Mechanisms in Radiation Biology, Proceedings of the N.A.T.O. Advanced Study Ins, Portmeirion 1967*, (ed. G.O. Philips), p.425. London: Academic Press.

Helmstetter, C.E. & Uretz, R.B. 1963. X-ray and ultraviolet sensitivity of synchronously dividing *E.coli*. *Biophys.J.* 3:35.

Helmstetter, C.E. & Pierucci 1968. Cell division during inhibition of deoxyribonucleic acid synthesis in *E.coli*. *J.Bacteriol.* 95:1627.

Hershey, A.D., Burgi, E. & Streisinger, G. 1958. Genetic recombination between phages in the presence of chloramphenicol. *Virology* 6:287.

Hofstad, T. 1979. Serological responses to antigens of *Bacteroidaceae*. *Microbiol.Rev.* 43:103.

Holdeman, L.V. & Moore, W.E.C. 1972. *Anaerobe Laboratory Manual.* Blacksburg: Virginia Polytechnic Institute and State University Anaerobe Laboratory.

Holdeman, L.V. & Moore, W.E.C. 1974. Family I. *Bacteroidaceae pribram* 1933. In *Bergey's manual of determinative bacteriology*, (eds R.E. Buchanan & N.E. Gibbons), 8th ed. p.384. Baltimore: Williams & Wilkins Co.

Holland, I.B. 1975. Physiology of colicin action. *Adv.Microbiol.Physiol.* 12:55.

Holliday, R. 1956. A new method for the identification of biochemical mutants of micro-organisms. *Nature, London* 178:987.

Hopwood, D.A. The Isolation of mutants. In *Methods in Microbiology* (eds J.R. Norris & D.W. Ribbons), Vol. 3A, p.364. London and New York: Academic Press.

Howard-Flanders, P. & Theriot, L. 1962. A method for selecting radiation-sensitive mutants of *E.coli*. *Genetics* 47:1219.

Howard-Flanders, P., Simson, E. & Theriot, L. 1964a. The excision of thymine dimers from DNA, filament formation and sensitivity to ultraviolet light in *E.coli* K-12. *Mutat.Res.* 1:219.

Howard-Flanders, P., Simson, E. & Theriot, L. 1964b. A locus that controls filament formation and sensitivity to radiation in *E.coli* K-12. *Genetics* 49:237.

Howard-Flanders, P. & Boyce, R.P. 1966. DNA repair and genetic recombination: Studies on mutants of *E.coli* defective in these processes. *Radiat.Res.* 6:156.

Howard-Flanders, P., Boyce, R.P. & Theriot, L. 1966. Three loci in *E.coli* K-12 that control the excision of pyrimidine dimers and certain other mutagen products from DNA. *Genetics* 53:1119.

Howard-Flanders, P., Rupp, W.D., Wilkins, B.M. & Cole, R.S. 1968. DNA replication and recombination after UV irradiation. *Cold Spring Harbor Symp.Quant.Biol.* 33:195.

Howard-Flanders, P. & Lin, P.F. 1973. Genetic recombination induced by DNA cross-links in repressed phage λ . *Genetics* 73:85.

Howarth, S. 1965. Resistance to the bactericidal effect of UV radiation conferred on entero-bacteria by the colicin factor Col I. *J.Gen.Microbiol.* 40:43.

Hungate, R.E. 1969. A roll-tube method for the cultivation of strict anaerobes. In *Methods in Microbiology* (eds J.R. Norris & D.W. Ribbons), Vol. 3B, p.117. London: Academic Press.

Huskey, R.J. 1969. Multiplicity reactivation as a test for recombination function. *Science* 164:319.

Ikeda, H. & Tomizawa, J-I. 1968. Prophage P1, and extrachromosomal replication unit. *Cold Spring Harbor Symp.Quant.Biol.* 33:791.

Ilno, T. 1977. Genetics of structure and function of bacterial flagella. *Ann.Rev.Genet.* 11:161.

Iyer, V.N. & Rupp, W.D. 1971. Usefulness of benzolated naptholated D.E.A.E. celluloses to distinguish and fractionate double stranded DNA bearing different extents of single stranded regions. *Biochim. Biophys.Acta* 228:117.

Jacob, F. & Wollman, E.L. 1953. Induction of phage development in lysogenic bacteria. *Cold Spring Harbor Symp.Quant.Biol.* 18:101.

Jacob, F. & Wollman, E.L. 1955. Étude genetique d'un bactériophage dempéré d'*E.coli*. III Effet du rayonnement ultraviolet sur la recombinaison génétique. *Ann.Inst.Pasteur.* 88:724.

Jagger, J.1958. Photoreactivation. *Bacteriol.Review* 22:99.

Jagger, J. 1960. Photoprotection from ultraviolet killing in *E.coli* B. *Radiat.Res.* 13:521.

Jagger, J., Wise, W.C. & Stafford, R.S. 1964. Delay in growth and division induced by near ultraviolet radiation in *E.coli* B and its role in photoprotection and liquid holding recovery. *Photochem.Photobiol.* 3:11.

Jagger, J. 1967. *Introduction to research in ultraviolet photobiology.* Englewood Cliffs: Prentice Hall Inc.

Johnson, J.L. 1978. Taxonomy of the *Bacteroides* I DNA homologies among *Bacteroides fragilis* and other saccharolytic *Bacteroides* species. *Int.J.Syst.Bacteriol.* 28:245.

Johnson, J.L. & Ault, D.A. 1978. Taxonomy of the *Bacteroides*. II. Correlation of phenotypic characteristics with DNA homology grouping for *Bacteroides fragilis* and other saccharolytic *Bacteroides* species. *Int.J.Syst.Bacteriol.* 28:257.

Jones, C.W., Brice, J.M, Wright, V. & Ackrell, B.A.C. 1973. Respiratory protection of a nitrogenase in *Azotobacter vinelandii*. *F.E.B.S. letters* 29:77.

Jones, L.M., McDuff, C.R. & Wilson, J.B. 1962. Phenotypic alterations in the colonial morphology of *Brucella abortus* due to a phage carrier-state. *J.Bacteriol.* 83:860.

Jones, R.N. & Fuchs, P.C. 1976. Identification and antimicrobial susceptibility of 250 *Bacteroides fragilis* subspecies by broth dilution methods. *Antimicrob.Agents Chemother.* 9:719.

Kantor, G.J. & Deering, R.A. 1966. Ultraviolet radiation studies of filamentous *E.coli* B. *J.Bacteriol.* 92:1062.

Kasper, D.L. 1976. The polysaccharide capsule of *Bacteroides fragilis* subspecies *fragilis:* Immunochemical and morphologic definition. *J.Infect.Dis.* 153:79.

Kasper, D.L. 1979. Virulence factors of anaerobic bacteria: An overview. *Rev.Infect.Dis.* 1:246.

Kasper, D.L., Hayes, M.E., Reinap, B.G., Craft, F.O., Onderdonk, A.B. & Polk, B.F. 1977. Isolation and identification of encapsulated strains of *Bacteroides fragilis*. *J.Infect.Dis.* 136:75.

Kasper, D.L., Onderdonk, A.B. & Bartlett, J.G. 1978. Quantitative determination of the antibody response to the capsular polysaccharide of *Bacteroides fragilis* in an animal model of intraabdominal abscess formation. *J.Infect.Dis.* 136:789.

Kasper, D.L., Onderdonk, A.B., Polk, B.F. & Bartlett, J.G. 1979. Surface antigens as virulence factors in infection with *B.fragilis*. *Rev.Infect.Dis.* 1:278.

Kawakami, M. & Landman, O.E. 1968. Nature of the carrier state of bacteriophage SP-10 in *B.subtilis*. *J.Bacteriol.* 95:1804.

Kellenberger, G. & Weigle, J. 1958. Etude au moyen des rayons ultraviolet de l'interaction entre bactériophage tempéré et bactérie hôte. *Biochim.Biophys.Acta* 30:112.

Keller, R. & Traub, N. 1974. The characterisation of *Bacteroides fragilis* bacteriophages recovered from animal sera: Observations on the nature of *Bacteroides* phage carrier cultures. *J.Gen.Virol.* 24:179.

Kellogg, D.S., Cohen, I.R., Norins, L.C., Schroeter, A.L. and Reising, G. 1968. *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months *in vitro*. *J.Bacteriol.* 96:596.

Kelly, R.B., Atkinson, M.R., Huberman, J.A. & Kornberg, A. 1969. Excision of thymine dimers and other mismatched sequences by DNA polymerase of *Escherichia coli*. *Nature* 224:495.

Kelner, A. 1949. Photoreactivation of UV irradiated *E.coli* with special reference to the dose reduction principle and to UV-induced mutations. *J.Bact.* 58:511.

Kelner, A. 1953. Growth, respiration and nucleic acid synthesis in UV irradiated and in photoreactivated *E.coli*. *J.Bacteriol.* 65:252.

Kislak, J.W. 1972. The susceptibility of *Bacteroides fragilis* to 24 antibiotics. *J.Infect.Dis.* 125:295.

Kleczkowski, A. 1951. Effects of non-ionizing radiations on viruses. *Adv.Virus.Res.* 4:191.

Kneser, H. 1968. Relationship between K-reactivation and UV-reactivation of bacteriophage λ . *Virology* 36:303.

Kos, E., Drakulič, M. & Brdar, B. 1965. Responses of various strains of *E.coli* to inhibition of some biosynthetic processes after ultraviolet irradiation. *Nature* 205:1125.

Kubitschek, H.E. Finney, A.C. & Krisch, R.E. 1973. Constancy of the UV sensitivity of *E.coli* during the cell cycle. *Photochem.Photobiol.* 18:365.

Kushner, S.R., Shepard, J., Edwards, G. & Marples, V.F. 1978. *uvr* D, *uvr* E and *rec* L represents a single gene. In *DNA Repair Mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C.F. Fox). New York: Academic Press.

Lai, C.H. & Listgarten, M.A. 1978. Ultrastructure characteristics of *Bacteroides melaninogenicus* ss. *melaninogenicus*, *asaccharolyticus*, *intermedius*. *J.Dent.Res.* 57A:317.

Leach, P.A., Bullen, J.J. & Grant, I.D. 1971. Anaerobic CO_2 cabinet for the cultivation of strict anaerobes. *Appl.Microbiol.* 22:824.

Lederberg, J & Lederberg, E.M. 1952. Replica plating and indirect selection of bacterial mutants. *J.Bacteriol.* 63:399.

Lehrbach, P.R., Kung, A.H.C. & Lee, B.T.O. 1978. R plasmids which alter UV light-sensitivity and enhance UV light-induced mutability in *Pseudomonas aeruginosa.* *J.Gen.Microbiol.* 108:119.

Lenov, D., Salmon, J. & Elad, D. 1973. UV- and gamma-ray-induced reactions of nucleic acids constituents with alcohol on the sensitivity of these reactions for purines. *Photochem.Photobiol.* 17:465.

Lev, M., Keudell, K.C. & Milford, A.F. 1971. Succinate as a growth factor for *Bacteroides melaninogenicus.* *J.Bacteriol.* 108:175.

Li, K., Barksdale, L. & Garmise, L. 1961. Phenotypic alterations associated with the bacteriophage carrier state of *Shigella dysenteriae.* *J.Gen.Microbiol.* 24:355.

Lindahl, T., Karran, P. & Riazuddin, S. 1978. DNA glycosylases of *E.coli.* In *DNA Repair Mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C.F. Fox). New York: Academic Press.

Lindberg, A.A., Berthold, P., Nord, C.E. & Weintraub, A. 1979. Encapsulated strains of *Bacteroides fragilis* in clinical specimens. *Med.Microbiol. Immunol.* 167:29.

Loesche, W.J. 1969. Oxygen sensitivity of various anaerobic bacteria. *Appl.Microbiol.* 18:723.

Luria, S.E. & Delbrück, M. 1942. Interference between bacterial viruses. II Interference between inactivated bacterial viruses and active viruses of the same strain and of a different strain. *Arch.Biochem.* 1:207.

Luria, S.E. 1944. A growth-delaying effect of UV radiation on bacterial viruses. *Proc.Natl.Acad.Sci.USA.* 30:393.

Luria, S.E. 1947. Reactivation of irradiated bacteriophage by transfer of self-reproducing units. *Proc.Nat.Acad.Sci.USA.* 33:253.

Luria, S.E. 1975. Colicins and the energetics of cell membranes. *Scient.Am.* 233:30.

Luria, S.E. & Dulbecco, R. 1949. Genetic recombinations leading to production of active bacteriophage from ultraviolet inactivated bacteriophage particles. *Genetics* 34:93.

Luria, S.E., Adams, J.N. & Ting, R.C. 1960. Transduction of lactose-utilizing ability among strains of *E.coli* and *S.dysenteriae* and the properties of the transducing phage particles. *Virology* 12:348.

McCord, J.M. & Fridovich, I. 1969. Superoxide dismutase: an enzymatic function for erythrocuprein. *J.Biol.Chem.* 244:6049.

McCord, J.M., Keele, B.B. & Fridovich, I. 1971. An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. *Proc.Natl.Acad.Sci.USA.* 68:1024.

McEntree, K. 1978. Studies of the *rec* A and *lec* A genes of *E.coli* K-12. In *DNA repair mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C.F. Fox), p.349. New York, San Fransisco, London: Academic Press.

McLaren, A.D. & Shugar, D. 1964. *Photochemistry of proteins and nucleic acids.* Pergamon.

MacPhee, D.G. 1972. Effect of an R factor on resistance of *Salmonella typhimurium* to radiation and chemical treatment. *Mutat.Res.* 14:450.

Mancini, C. & Behme, R.J. 1977. Transfer of multiple antibiotic resistance from *Bacteroides fragilis* to *E.coli.* *J.Infect.Dis.* 136:597.

Markovitz, A. 1964. Regulatory mechanisms for synthesis of capsular polysaccharide in mucoid mutants of *E.coli* K-12. *Proc.Natl.Acad. Sci. USA.* 51:239.

Markovitz, A. & Baker, B. 1967. Suppression of radiation sensitivity and capsular polysaccharide synthesis in *E.coli* K-12 by ochre suppressors. *J.Bacteriol.* 94:388.

Martin, W.J. 1971. Practical method for isolation of anaerobic bacteria in the clinical laboratory. *Appl.Microbiol.* 22:1168.

Martin, W.J., Gardner, M. & Washington, J.A. 1972. *In vitro* antimicrobial susceptibility of anaerobic bacteria isolated from clinical specimens. *Antimocrob.Agents Chemother.* 1:148.

Mašek, F., Štefunková, E. & Sedliaková, M. 1971. Effect of amino acid starvation on the degradation of DNA in *E.coli* B/r after UV irradiation. *FEBS Letters* 13:10.

Mathias, R.G., Harding, G.K.M., Gurwith, M.J., Ströer, H.G., Sigurdson, E., Gratton, C.A. & Roland, A.R. Bacteremia due to *Bacteroidaceae:* A review of 92 cases. *J.Infect.Dis.* 135:69.

Mayr-Harting, A., Hedges, A.J. & Berkeley, R.C.W. 1972. Methods for studying bacteriocins. In *Methods in Microbiology* (eds J.R. Norris & D.W. Ribbons), Vol. 7A, p.315. New York: Academic Press.

Metzger, K. 1964. On the dark reactivation mechanism in ultraviolet irradiated bacteria. *Biochem.Biophys.Res.Commun.* 15:101.

Meyn, M.S., Rossman, T., Gottlieb, P. & Troll, W. 1978. The role of proteases in SOS regulation. In *DNA Repair Mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C.F. Fox), pp.379. New York, San Fransisco, London: Academic Press.

Meynell, G.G. & Meynell, Elinor. 1970. *Theory and Practice in Experimental Bacteriology.* 2nd ed. Cambridge University Press.

Michalke, H. & Bremer, H. 1969. RNA synthesis in *E.coli* after irradiation with UV light. *J.Mol.Biol.* 41:1.

Miles, D.O., Dyer, J.K. & Wong, J.C. 1976. Influence of amino acids on the growth of *Bacteroides melaninogenicus*. *J.Bacteriol.* 127:899.

Mitsuhashi, S. 1965. Transmissible drug-resistance factor R. *Gunma J. Med.Sci.* 14:169.

Miura, A. & Tomizawa, J.I. 1968. Studies on radiation-sensitive mutants of *E.coli*. III. Participation of the *rec* system in induction of mutation by ultraviolet irradiation. *Mol.Gen.Genet.* 103:1.

Mizuuchi, K., O'Dea, M.H. & Gellert, M. 1978. DNA gyrase: subunit structure and ATPase activity of the purified enzyme. *Proc.Natl. Acad.Sci.USA.* 75:5960.

Monk, M., Peacey, M. & Gross, J.D. 1971. Repair of damage induced by UV light in DNA polymerase-defective *E.coli* cells. *J.Mol.Biol.* 58:623-630.

Moodie, H.L. & Woods, D.R. 1973a. Anaerobic R factor transfer in *Escherichia coli*. *J.Gen.Microbiol.* 76:437.

Moodie, H.L. & Woods, D.R. 1973b. Isolation of obligate anaerobic faecal bacteria using an anaerobic glove cabinet. *S.A.Med.J.* 47:1739.

Moore, W.E.C. 1966. Techniques for routine culture of fastidious anaerobes. *Int.J.Syst.Bacteriol.* 16:173.

Moore, W.E.C., Cato, E.P. & Holdeman, L.V. 1969. Anaerobic bacteria of the gastrointestinal flora and their occurrence in clinical infections. *J.Infect.Dis.* 119:641.

Moore, W.E.C. & Holdeman, L.V. 1972. Identification of anaerobic bacteria. *Am.J.Clin.Nutr.* 25:1306.

Morris, J.G. 1975. The physiology of obligate anaerobiosis. *Adv. Microbial.Physiol.* 12:169.

Morris, J.G. 1976. Oxygen and the obligate anaerobe. *J.Appl.Bact.* 40:229.

Morris, J.G. & O'Brien, R.W. 1971. Oxygen and *Clostridia:* a review. In *Spore Research* (eds A.N. Barker, G.W. Gould & J. Wolf), p.1. New York & London: Academic Press.

Morseley, B.E.B. 1968. The repair of damaged DNA in irradiated bacteria. *Advan.Microb.Physiol.* 2:173.

Mortelmans, K. & Friedberg, E.C. 1972. DNA repair in phage T4: observations on the role of the X and V genes and of host factors. *J.Virol.* 10:730.

Morton, R.A. & Haynes, R.H. 1969. Changes in the ultraviolet sensitivity of *E.coli* during growth in batch cultures. *J.Bacteriol.* 97:1379.

Moss, S.H. & Daives, D.J.G. 1974. Interrelationship of repair mechanisms in ultraviolet-irradiated *E.coli*. *J.Bacteriol.* 120:15.

Mossie, K.G., Jones, D.T., Robb, F.T. & Woods, D.R. 1979. Characterisation and mode of action of a bacteriocin produced by a *Bacteroides fragilis* strain. *Antimicrob.Agents.Chemother.* In press (Dec. issue).

Murray, P.R. & Rosenblatt, J.E. 1972. Antibiotic resistance in anaerobic bacteria. *Antimicrob.Ag.Chemother.* 11:605.

Nacescu, N., Brandis, H. & Werner, H. 1972. Isolation of two *Bacteroides fragilis* phages from sewage and detection of lysogenic *B.fragilis* strains. *Zbl.Bakt.Hyg.*, I. Abt. Orig. A. 219:522.

Nastro, L.S. & Finegold, S.M. 1972. Bactericidal activity of five antimicrobial agents against *Bacteroides fragilis*. *J.Infect.Dis.* 126:104.

O'Brien, R.W. & Morris, J.G. 1971. Oxygen and growth and metabolism of *Clostridium acetobutylicum*. *J.Gen.Microbiol.* 68:307.

Ogawa, H., Shimada, K. & Tomizawa, J.I. 1968. Studies on radiation-sensitive mutants of *E.coli*. I. Mutants defective in the repair synthesis. *Mol.Gen.Genet.* 101:227.

Okagaki, H. 1960. Effect of chloramphenicol on the survival of *E.coli* irradiated with UV light. *J.Bact.* 79:277.

Onderdonk, A.B., Johnston, J., Mayhew, J.W. & Gorbach, S.L. 1976. Effect of dissolved O_2 and Eh on *Bacteroides fragilis* during continuous culture. *Appl.Environ.Microbiol.* 31:168.

Onderdonk, A.B., Kasper, D.L., Cisneros, R.L. & Bartdett, J.G. 1977. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: Comparison of the pathogenic potential of encapsulation on unencapsulated strains. *J.Infect.Dis.* 136:82.

Ono, J. & Shimazu, Y. 1966. Ultraviolet reactivation of bacteriophage containing a single-stranded DNA as a genetic element. *Virology* 29:295.

Parisi, T. & Talbot, H.W. 1974. Improved rapid plate method for the isolation of bacteriophages from lysogenic bacteria. *J.Appl.Microbiol.* 38:503.

Patrick, M.H., Haynes, R.H. & Uretz, R.B. 1964. Dark recovery phenomena in yeast: I. Comparative effects with various inactivating agents. *Radiat.Res.* 21:144.

Phillips, S.L., Person, S. & Jagger, J. 1967. Division delay induced in *E.coli* by near-ultraviolet radiation. *J.Bacteriol.* 94:165.

Podhaisky, C. & Reinhold, L. 1970. Bacteriocinbildung und bacteriocin sensibilitat bei *Bacteroides fragilis* und *Bacteroides thetaiotaomicron* aus klinischem material.*Zbl.Bakt.Hyg.* I. Abt.Orig.A. 219:522.

Polk, B.F. & Kasper, D.L. 1977. *Bacteroides fragilis* subspecies in clinical isolates. *Ann.Intern.Med.* 86:569.

Poxton, I.R. & Sutherland, I.W. 1976. Isolation of rough mutants of *Klebsiella aerogenes* and their synthesis of polysaccharides. *J.Gen.Microbiol.* 96:195.

Prévot, A.R., Vieu, J.F., Thouvenot, H. & Brault, G. 1970. Etude de *Ristella pseudoinsolita* et de ses bacteriophages essai de lysotypie. *Bull.Acad.Nat.Med.* 154:681.

Privitera, G., Sebald, M. & Fayolle, F. 1979a. Common regulatory mechanism of expression and conjugative ability of a tetracycline resistance plasmid in *Bacteroides fragilis*. *Nature* 278:657.

Privitera, G., Bublanchet, A. & Sebald, Madeleine 1979b. Transfer of multiple antibiotic resistance between subspecies of *Bacteroides fragilis*. *J.Infect.Dis.* 139:97.

Putnam, F.W., Kozloff, L.M. & Neill, J.C. 1949. Biochemical studies of virus reproduction: I. Purification and properties of *E.coli* bacteriophage T6. *J.Biol.Chem.* 179:303.

Quinto, G. 1962. Nutrition of five *Bacteroides* strains. *J.Bacteriol.* 84:559.

Quinto, G. 1966. Amino-acid and vitamin requirements of several *Bacteroides* strains. *Appl.Microbiol.* 14:1022.

Quinto, G. & Sebald, M. 1964. Identification of three hemia-requiring *Bacteroides* strains. *Amer.J.Med.Technol.* 30:318.

Radding, C.M. 1973. Molecular mechanisms in genistic recombination. *Ann.Rev.Genet.* 7:87.

Radman, M. 1974. Phenomenology of an inducible mutagenic repair pathway in *E.coli:* SOS repair hypothesis. In *Molecular and Environmental Aspects of Mutagenesis, Sixth International Conference on Environmental Toxicity, June 4-6, 1973*, p.128. Springfield: Charles C. Thomas.

Radman, M. 1975. SOS repair hypothesis. In *Molecular Mechanisms for Repair of DNA* (eds P.C. Hanawalt & R.B. Setlow), p.355. New York: Plenum Press.

Radman, M. 1977. A molecular mechanism for site-specific recombination. In *Colloques Internationaux du C.N.R.S.* No. 256, p.293. Paris: C.N.R.S.

Radman, M., Cordone, L., Krsmanovic-Simic, D. & Errera, M. 1970. Complementary action of recombination and excision in the repair of UV irradiation damage to DNA. *J.Mol.Biol.* 49:203.

Radman, M. & Devoret, R. 1971. UV reactivation of bacteriophage λ. in excision repair deficient host: independence of red functions and attachment regions. *Virology* 43:504.

Rahn, R.O., Landry, L. & Carrier, W.L. 1974. Formation of chain breaks and thymine dimers in DNA upon photosensitization at 313 nm with acetophenone, acetone, or benzophenone. *Photochem.Photobiol.* 19:75.

Ramabhadran, T.V., Fossum, T. & Jagger, J. 1975. The chromophore and target for near-UV induced growth delay in *E.coli*. *Abstr.Amer.Soc. Photobiol.* 3:92.

Ravin, V.K. & Shulga, M.G. 1970. Evidence for extrachromosomal location of prophage N15. *Virology* 40:800.

Reeves, P. 1972. The bacteriocins. *Molecular Biology, Biochemistry and Biophysics*. New York: Springer-Verlag/Chapman & Hall.

Robbins, M.L., Somer, A.M. & Parr, L.W. 1957. Variation in the antibiotic producing bacterial flora of the human intestinal tract. *J.Bacteriol.* 74:377.

Roberts, J.W. & Roberts, C.W. 1975. Protolytic cleavage of bacteriophage lambda repressor in induction. *Proc.Natl.Acad.Sci.USA*. 72:147.

Roberts, J.W., Roberts, C.W. & Mount, D.W. 1977. Inactivation and proteolytic cleavage of phage λ repressor *in vitro* in an ATP-dependent reaction. *Proc.Natl.Acad.Sci.USA*. 74:2283.

Roberts, J.W., Roberts, C.W. & Craig, N.L. 1978. *E.coli rec* A gene product inactivates phage λ repressor.*Proc.Natl.Acad.Sci.USA*. 75:4714.

Roberts, R.B. & Aldous, E. 1949. Recovery from UV irradiation in *E.coli*. *J.Bacteriol.* 57:363.

Rolfe, R.D., Hentges, D.J., Barrett, J.T. & Campbell, B.J. 1977. Oxygen tolerance of human intestinal anaerobes. *Am.J.Clin.Nutr.* 30:1762.

Romig, W.R. & Brodetsky, A.M. 1961. Isolation and preliminary characterization of bacteriophages for *Bacillus subtilis*. *J.Bacteriol.* 82:135.

Rosebury, R. 1962. *Micro-organisms Indigenous to Man*. New York: McGraw-Hill.

Rossman, T., Meyn, M.S. & Troll, W. 1975. Effects of sodium arsenite on the survival of UV irradiated *E.coli* inhibition of *rec* A dependent function. *Mutat.Res.* 30:157.

Rossman, T.G., Meyn, M.S. & Troll, W. 1977. Effects of arsenite on DNA repair in *E.coli*. *Environmental Health Perspectives* 10:48.

Rudé, J. & Alper, T. 1972. Changes in UV survival curves of *E.coli* B/r concomitant with changes in growth conditions. *Photochem.Photobiol.* 15:51.

Rudé, J.M., & Doudney, C.O. 1973. Relation between survival and deoxyribonucleic acid replication in ultraviolet-irradiated resistant and sensitive strains of *E.coli* B/r. *J.Bacteriol.* 113:1161.

Rupert, C.S. 1962. Photoenzymatic repair of UV damage in DNA. *J.Gen.Physiol.* 45:703.

Rupert, C.S. 1964. Photoreactivation of ultraviolet damage. In *Photophysiology* (ed. A.C. Giese), Vol. 11, p.283. New York: Academic Press.

Rupert, C.S. 1975. Enzymatic photoreactivation: Overview. In *Molecular Mechanisms for Repair of DNA* (eds P.C. Hanewalt & R.B. Setlow), Part A, p.73. New York & London: Plenum Press.

Rupert, C.S. & Harm, W. 1966. Reactivation after photobiological damage. In *Advances in Radiation Biology* (eds L.G. Augenstein, R. Mason & M.R. Zelle), Vol. 11, p.1. New York: Academic Press.

Rupert, C.S., Goodgal, S.H. & Herriott, R.M. 1958. Photoreactivation *in vitro* of ultraviolet inactivated *Hemophilus influenzae* transforming factor. *J.Gen.Physiol.* 41:451.

Rupp, W.D. & Howard-Flanders, P. 1968. Discontinuities in the DNA synthesis in an excision-defective strain of *E.coli* following UV irradiation. *J.Mol.Biol.* 31:291.

Rupp, W.D., Wilde, C.E., Reno, D.L. & Howard-Flanders, P. 1971. Exchanges between DNA strands in UV-irradiated *E.coli*. *J.Mol.Biol.* 61:25.

Sabiston, C.B. & Cohl, M.E. 1969. Bacteriophage virulent for species of the genus *Bacteroides*. *J.Dent.Res.* 48:599.

Saedler, H., Reif, H.J., Hu, S. & Davidson, D. 1974. IS2 a genetic element for turn-off and turn-on of genetic activity in *E.coli*. *Molec.Gen. Genet.* 132:265.

Salyers, A.A., Vercellotti, J.R., West, S.E.H. & Wilkins, T.D. 1977. Fermentation of mucia and plant polysaccharides by strains of *Bacteroides* from the human colon. *Appl.Environ.Microbiol.* 33:319.

Sancar, A. & Rupert, C.S. 1978. A general method for isolation of repair deficient mutants. In *DNA Repair Mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C.F. Fox), p.101. New York: Academic Press.

Sancar, A. & Rupert, C.S. 1979. Penicillin selection of *E.coli* deoxyribonucleic acid repair mutants. *J.Bact.* 138:779.

Sapico, F.L., Kwok, Y., Sutter, V.L. & Finegold, S.M. 1972. Standardised antimicrobial disc susceptibility testing of anaerobic bacteria: *in vitro* susceptibility of *Clostridium perfringens* to nine antibiotics. *Antimicrob.Agents.Chemother.* 2:320.

Sauerbier, W. 1964. Inhibition of host cell reactivation in phate T1 by caffeine. *Biochem.Biophys.Res.Commun.* 14:340.

Schaedler, R.W. & Costello, R. 1963. The development of the bacterial flora in the gastrointestinal tract of mice. *J.Exp.Meg.* 122:59.

Schaller, K. & Nomura, M. 1976. Colicin E2 is a DNA endonuclease. *Proc.Natl.Acad.Sci.USA.* 73:3989.

Scott McDaniel, L. & Hill, W.E. 1979. Sensitivity of recombination deficient mutants of *E.coli* to nalidixic acid. *Abstr.Annu.Meet. Am.Soc.Microbiol.* 79:123.

Sears, H.J., Brownlee, I. & Uchiyama, J.K. 1950. Persistence of individual strains of *Escherichia coli* in the intestinal tract of man. *J.Bacteriol.* 59:293.

Sebald, M. & Costilow, R.N. 1975. Minimal growth requirements for *Clostridia perfringens* and isolation of auxotrophic mutants. *Appl.Microbiol.* 29:1.

Sedgwick, S.G. & Bridges, B.A. 1974. Requirements for either DNA polymerase I or DNA polymerase III in post-replication repair in excision-proficient *E.coli*. *Nature (Lond)* 249:348.

Sedliaková, M., Mašek, F., Brozmanová, J., Maskova, L., Bugán, J. & Slezáriková, V. 1973. Depression of thymine dimer excision without an adequate effect on survival in *E.coli* B/r *Hcr+* cells. *Neoplasma* 20:499.

Seeberg, E. & Rupp, W.D. 1975. Effect of mutations in *lig* and *pol* A on UV-induced strand cutting in a *uvr* C strain of *E.coli*. In *Molecular Mechanisms for the Repair of DNA* (eds P.C. Hanawalt & R.B. Setlow). London: Pergamon.

Sekiguchi, M., Shimizu, K., Sato, K., Yasuda, S. & Onshima, S. 1975. Enzymatic mechanism of excision-repair in T4 infected cells. In *Molecular Mechanisms for Repair of DNA* (eds P.C. Hanawalt & R.B. Setlow), p.135. New York: Plenum Press.

Setlow, J.K. 1966. The molecular basis of biological effects of ultraviolet radiation and photoreactivation. In *Curr.Top.Radiat. Res.* 2:195, (eds M. Ebert & A. Howard). Amsterdam: North-Holland Pub.Co.

Setlow, R.B. 1964. Physical changes and mutagenesis. *J.Cell.Comp. Physiol.* 64:51.

Setlow, R.B. 1967a. Steps in the repair of DNA: fact and fancy. *Brookhaven Symposia in Biology* 20:1.

Setlow, R.B. 1967b. The effects of ultraviolet radiation and photoreactivation. In *Comprehensive Biochemistry, Photobiology, Ionizing Radiation* (eds M. Florkin & E.H. Stotz), Vol. 27, p.157. Amsterdam: Elsevier.

Setlow, R.B. 1968. The photochemistry, photobiology and repair of polynucleotides. *Progr.Nucl.Acid.Res.* 8:247.

Setlow, R.B., Swenson, P.A. & Carrier, W.L. 1963. Thymine dimers and inhibition of DNA synthesis by UV irradiation of cells. *Science* 142:1468.

Setlow, R.B. & Carrier, W.L. 1964. The disappearance of thymine dimers from DNA: an error-correcting mechanism. *Proc.Natl.Acad.Sci.USA.* 51:226.

Setlow, R.B. & Carrier, W.L. 1966. Pyrimidine dimers in ultraviolet irradiated DNA. *J.Mol.Biol.* 17:237.

Setlow, R.B. & Carrier, W.L. 1967. Formation and destruction of pyrimidine dimers in polynucleotides by ultraviolet irradiation in the presence of proflavine. *Nature* 213: 906.

Setlow, R.B. & Carrier, W.L. 1968. The excision of pyrimidine dimers *in vivo* and *in vitro*. In *Replication and Recombination of Genetic Material* (eds W.J. Peacock & R.D. Brock), p.134. Canberra: Australian Academy of Science.

Shimada, K., Ogawa, H. & Tomizawa, J.I. 1968. Studies on radiation-sensitive mutants of *E.coli*. II. Breakage and repair of UV irradiated intracellular DNA of phage lambda. *Mol.Gen.Genet.* 101:245.

Siccardi, A.G. 1969. Effect of R factors and other plasmids on ultraviolet susceptibility and host cell reactivation property of *E.coli*. *J.Bacteriol.* 96:198.

Siddiqui, A., Hart, E., Mandagere, U. & Goldberg, I.D. 1974. Rapid plate method for isolation of lysogenic bacteriophages. *Appl.Microbiol.* 27:278.

Silver, R.P., Chase, D.G., Tally, F.P. & Gorbach, S.L. 1975. Bacteriophage associated spherical bodies in *Bacteroides fragilis*. *J.Virol.* 15:894.

Silverman, M. & Simon, M.I. 1977. Bacterial flagella. *Ann.Rev.Microbiol.* 31:397.

Smith, H.W. & Huggins, M.B. 1976. Further observations on the association of the colicine V plasmid of *Escherichia coli* with pathogenicity and with survival in the alimentary tract. *J.Gen.Microbiol.* 92:335.

Smith, L.D. & Holdeman, L.V. 1968. *The Pathogenic Anaerobic Bacteria.* Springfield, Illinois: Charles C. Thomas.

Smith, K.C. 1964. Photochemistry of the nucleic acids, In *Photophysiology* (ed. A.C. Giese), Vol. 2, p.329. New York: Academic Press.

Smith, K.C. 1969. DNA synthesis in sensitive and resistant mutants of *B.coli* B after UV irradiation. *Mutat.Res.* 8:481.

Smith, K.C. 1976. The role of nucleic acid addition reactions. In *Aging, Carcinogenesis and Radiation Biology* (ed. K.C. Smith). New York: Plenum Press.

Smith, K.C. 1978a. Molecular aspects of the interaction of far-ultraviolet radiation with living matter, In *International Symposium on Current Topics in Radiobiology and Photobiology*, (ed. R.M. Tyrrell). Rio de Janeiro: Academia Brasileira de Ciências.

Smith, K.C. 1978b. Multiple pathways of DNA repair in bacteria and their roles in mutagenesis. *Photochem.Photobiol.* 28:121.

Smith, K.C., Hodgkins, B. & O'Leary, M.E. The biological importance of UV light induced DNA-protein crosslinks in *E.coli* 15 TAU. *Biochim. Biophys.Acta* 114:1.

Smith, K.C., & Meun, D.H.C. 1970. Repair of radiation-induced damage in *Escherichia coli*. I. Effect of *rec* mutations on post-replication repair of damage due to ultraviolet radiation. *J.Mol.Biol.* 51:459.

Smith, K.C., Youngs, D.A. van der Schueren, E., Carlson, M. & Sargentini, N.J. 1978. Excision repair and mutagenesis are complex processes. In *DNA Repair Mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C.F. Fox), p.247. New York: Academic Press.

Sperling, J. & Havron, A. 1977. Protein-nucleic acid photo-interaction. *Photochem.Photobiol.* 26:661.

Sperry, J.F. & Wilkins, T.D. 1976. Arginine, a growth-limiting factor for *Eubacterium lentum*. *J.Bacteriol.* 127:780.

Stallions, D.R. & Curtiss, R. 1972. Bacterial conjugation under anaerobic conditions. *J.Bacteriol.* 111:294.

Stent, G.S. 1963. *Molecular Biology of Bacterial Viruses* (eds D.M. Whitaker, R. Emerson, D. Kennedy & G.W. Beadle). San Fransisco & London: W.H. Freeman & Company.

Stiffler, P.W., Keller, R. & Traub, N. 1974. Isolation and characterization of several cryptic plasmids from clinical isolates of *Bacteroides fragilis*. *J.Infect.Dis.* 130:544.

Stocker, B.A.D. 1949. Measurement of rate of mutation of flagella antigenic phase in *Salmonella typhimurium*. *J.Hyg.* 47:398.

Sutherland, B.M. 1978. Enzymatic photoreactivation of DNA. In *DNA Repair Mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C.F. Fox), p.113. New York: Academic Press.

Sutherland, B.M. & Sutherland, J.C. 1969. Mechanisms of inhibition of pyrimidine dimer formation in deoxyribonucleic acid by acridine dyes. *Biophys.J.* 9:292.

Sutter, V.L., Vargo, V.L., Finegold, S.M. & Bricknell, K.S. 1975. *Wadsworth Anaerobic Bacteriology Manual.* Los Angeles: The Regents of University of California.

Sutter, V.L. & Finegold, S.M. 1976. Susceptibility of anaerobic bacteria to 23 antimicrobial agents. *Antimicrob.Agents.Chemother.* 10:736.

Suzuki, K. & Iwama, Y. 1960. Recovery in the presence of chloramphenicol of *E.coli* B(H) irradiated with UV light. *Nature* 187:347.

Swenson, P.A. 1976. Physiological responses of *E.coli* to far-UV radiation. In *Photochemical and Photobiological Reviews* (ed. K.C. Smith) Vol. 1, p.269. New York: Plenum Press.

Swenson, P.A. 1978. Death of *E.coli* cells as a consequence of damage to DNA by far UV radiation. In *DNA Repair Mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C.F. Fox). New York: Academic Press.

Swenson, P.A. & Schenley, R.L. 1970a. Evidence for the control of respiration by DNA in untraviolet-irradiated *E.coli* B/r cells. *Mutat.Res.* 9:443.

Swenson, P.A. & Schenley, R.L. 1970b. Role of pyridine nucleotides in the control of respiration in ultraviolet-irradiated *E.coli* B/r cells. *J.Bact.* 104:1230.

Swenson, P.A. & Schenley, R.L. 1972. Death through respiratory failure of a fraction of ultraviolet-irradiated *E.coli* B/r cells. *J.Bact.* 111:658.

Swenson, P.A. & Schenley, R.L. 1974a. Respiration growth and viability of repair-deficient mutants of *E.coli* after UV irradiation. *Int.J. Radiat.Biol.* 25:51.

Swenson, P.A. & Schenley, R.L. 1974b. Evidence relating cessation of respiration, cell envelope changes, and death in UV-irradiated *E.coli* B/r cells. *J.Bact.* 117:551.

Swenson, P.A., Ives, J.E. & Schenley, R.L. 1975. Photoprotection of *E.coli* B/r: respiration growth, macromolecular synthesis and repair of DNA. *Photochem.Photobiol.* 21:235.

Swenson, R.M., Lorber, B., Michaelson, T.C. & Spaulding, E.H. 1974. The bacteriology of intraabdominal infections. *Arch.Surg.* 109:398.

Symonds, N.D. 1968. Chemical mutagenesis with 5-bromouracil. In *Experiments in Microbial Genetics* (eds R.C. Clowes & W. Hayes). Oxford & Edinburgh: Blackwell Scientific Publications.

Symonds, N. 1975. The repair of ultraviolet damage by phage T4: the role of the early phage genes. In *Molecular Mechanisms for Repair of DNA* (eds P.C. Hanawalt & R.B. Setlow), p.143. New York: Plenum Press.

Symonds, N., Heindl, H. & White, P. 1973. The chemical basis for a case of host induced modification in phage T2. *Mol.Gen.Genet.* 120:253.

Tait, R.C., Harris, A.L. & Smith, D.W. 1974. DNA repair in *Escherichia coli* mutants deficient in DNA polymerases I, II and/or III. *Proc.Natl.Acad.Sci.USA.* 71:675.

Takazoe, I., Tanaka, M. & Homma, T. 1971. A pathogenic strain of *Bacteroides melaninogenicus*. *Arch.Oral.Biol.* 16:817.

Tally, F.P., Stewart, P.R., Sutter, V.L. & Rosenblatt, J. 1975. Oxygen tolerance of fresh clinical anaerobic bacteria. *J.Clin.Microbiol.* 1:161.

Tally, F.P., Goldin, H.R., Jacobus, N.V. & Gorbach, S.L. 1977. Superoxide dismutase in anaerobic bacteria of clinical significance. *Infec. Immun.* 16:20.

Tally, F.P., Snydman, D.R., Gorbach, S.L. & Malamy, M.H. 1979. Plasmid-mediated, transferable resistance to clindamycin and erythromycin in *Bacteroides fragilis*. *J.Infec.Dis.* 139:83.

Tamimi, H.A., Hiltbrand, W. & Loercher, H. 1960. Some growth requirements of *B.fragilis*. *J.Bacteriol.* 80:472.

Tang, M. & Patrick, M.H. 1977a. Repair of UV damage in *E.coli* under non-growth conditions. *Photochem.Photobiol.* 26:247.

Tang, M. & Patrick, M.H. 1977b. The role of DNA polymerase I in liquid holding recovery of UN-irradiated *E.coli*. *Photochem.Photobiol.* 26:257.

Tang, M., Wang, T.V. & Patrick, M.H. 1979. DNA turnover in buffer-held *E.coli* and its effect on repair of UV damage. *Photochem.Photobiol.* 29:511.

Tessman, E.S. & Ozaki, T. 1959. Some unusual properties of the nucleic acid in bacteriophage S13 and ϕ X 174. *Virology* 7:263.

Tessman, E.S. & Ozaki, T. 1960. The interaction of phage S13 with UV irradiated host cells and properties of the ultraviolet irradiated phage. *Virology* 12:431.

Thadepalli, H., Gorbach, S.L. & Broido, P.N. 1973a. Abdominal trauma, anaerobes and antibiotics. *Surg.Gynecol.Obstet.* 137:270.

Thadepalli, H., Gorbach, S.L. & Keith, L. 1973b. Anaerobic infection of the female genital tract: bacteriologic and therapeutic aspects. *Am.J.Obstet.Gynecol.* 117:1034.

Thompson, L.A., Gates, D.M., Ingledew, W.M. & Jones, G.A. Use of the Hungate anaerobic technique in the isolation of phloroglucinol-negative mutants of *Coprococcus* species. *Appl.Environ.Microbiol.* 31:21.

Thorley, C.M. 1976. A simplified method for the isolation of *Bacteroides nodusus* from bovine foot-rot and studies on its colony morphology and serology. *J.Appl.Bacteriol.* 40:301.

Thorne, C.B. 1962. Transduction in *Bacillus subtilis.* *J.Bacteriol.* 83:106.

Thornton, G.F. & Cramer, J.A. 1971. Antibiotic susceptibility of *Bacteroides* species. *Antimicrob.Agents Chemother.* p.509.

Tinnell, W.H. & Macrina, F.L. 1976. Extra-chromosomal elements in a variety of strains representing the *Bacteroides fragilis* group of organisms. *Infect.Immun.* 14:955.

Tyrrell, R.M. 1973. Induction of pyrimidine dimers in bacterial DNA by 365 nm radiation. *Photochem.Photobiol.* 17:69.

Tyrrell, R.M., Moss, S.H. & Davies, D.J.G. 1972. The variation in UV sensitivity of four K-12 strains of *E.coli* as a function of their stage of growth. *Mutat.Res.* 16:1.

van Tassell, R.L. & Wilkins, T.D. 1978. Isolation of auxotrophs of *Bacteroides fragilis.* *Canad.J.Microbiol.* 24:1619.

Varel, V.H. & Bryant, M.P. 1974. Nitritional features of *Bacteroides fragilis* subsp. *fragilis.* *Appl.Microbiol.* 18:251.

Veillon, A. & Zuber, A. 1898. Recherches sur quelques microbes strictment anaérobies et leur role en pathologie. *Arch.Med.Exp.Anat.Pathol.* 10:517.

Vieu, J.F. 1964. Applications de la bacteriocinogenie et des bacteriocines. *Ann.Inst.Pasteur* (Paris) 197:93.

Wainfan, E., Mandel, L.R. & Borek, E. 1963. Differential inhibition of the synthesis of soluble and particulate RNA. *Biochem.Biophys.Res. Commun.* 10:315.

Walden, W.C. & Hentges, D.J. 1975. Differential effects of oxygen and oxidation-reduction potential on the multiplication of three species of anaerobic intestinal bacteria. *Appl.Microbiol.* 30:781.

Walker, J.R. & Pardee, A.B. 1968. Evidence for a relationship between deoxyribonucleic acid metabolism and spetum formation in *E.coli.* *J.Bacteriol.* 95:123.

Wang, S.Y. 1976. *Photochemistry and Photobiology of Nucleic Acids.* (ed. S.Y. Wang). New York: Academic Press.

Washington, J.A. 1971. Comparison of two commercially available media for detection of bacteremia. *Appl.Microbiol.* 22:604.

Weatherwax, R.S. 1956. Desensitization of *E.coli* to UV light. *J.Bacteriol.* 72:124.

Webb, R.B. & Petrusek, R.L. 1966. Oxygen effect in the protection of *E.coli* against UV inactivation and mutagenesis by acridine orange. *Photochem.Photobiol.* 5:645.

Webb, R.B. & Lorenz, J.R. 1970. Oxygen dependence and repair of lethal effects of near UV and visible light. *Photochem.Photobiol.* 12:283.

Webb, R.B. 1977. Lethal and mutagenic effects of near-UV radiation. In *Photochemical and Photobiological Reviews* (ed. K.C. Smith), Vol. 2, p.169. New York: Plenum Press.

Weigle, J.J. 1953. Induction of mutations in a bacterial virus. *Proc.Natl.Acad.Sci.USA.* 39:628.

Weigle, J.J. 1966. Story and structure of the λ transducing phage. In *Phage and the Origins of Molecular Biology* (eds J. Cairns, G.S. Stent & J.D. Watson), p.226. New York: Cold Spring Harbour Lab.

Welch, R.A., Jones, K.R. & Macrina, F.L. 1979. Transferable lincosamide-macrolide resistance in *Bacteroides.* *Plasmid* 2:261.

White, D.C., Bryant, M.P. & Caldwell, D.R. 1962. Cytochrome-linked fermentation in *Bacteroides ruminidola.* *J.Bacteriol.* 84:822.

Wilkins, T.D. & Hackman, A.S. 1974. Two patterns of neutral steroid conversion in the faeces of normal North Americans. *Cancer Res.* 34:2250.

Wilkins, T.D., Wagner, D.L., Veltri, B.J. & Gregory, E.M. 1978. Factors affecting production of catalase by *Bacteroides.* *J.Clin.Microbiol.* 8:553.

Wilkinson, J.F. 1958. The extracellular polysaccharides of bacteria. *Bact.Rev.* 22:46.

Willis, A.T. 1969. Techniques for the study of anaerobic, spore-forming bacteria. In *Methods in Microbiology* (eds J.R. Norris & D.W. Ribbons), Vol. 3b, p.79. London: Academic Press.

Wilson, G.S. & Miles, A.A. 1975. In *Topley and Wilson's Principles of Bacteriology and Immunity* p.1109. London: Edward Arnold.

Wilson, G.G., Young, K.K.Y. & Edlin, G.J. 1979. High-frequency generalised transduction by bacteriophate T4. *Nature* 280:80.

Wing, J.P. 1968. Transduction by phate P22 in a recombination-deficient mutant of *Salmonella typhimurium.* *Virology* 36:271.

Witkin, E.M. 1946. Inherited differences in sensitivity to radiation in *E.coli*. *Proc.Natl.Acad.Sci.USA.* 32:59.

Witkin, E.M. 1947. Genetics of resistance to radiation in *E.coli*. *Genetics* 32:221.

Witkin, E.M. 1959. Post-irradiation metabolism and the timing of ultraviolet-induced mutations in bacteria. In *Tenth International Congress of Genetics Montreal*, Vol. 1, p.280. Toronto: University of Toronto Press.

Witkin, E.M. 1961. Modification of mutagenesis initiated by ultraviolet light through post-treatment of bacteria with basic dyes. *J.Cell. Comp.Physiol.* 58:135.

Witkin, E.M. 1963. The effect of acriflavine on photoreversal of lethal and mutagenic damage produced in bacteria by UV light. *Proc.Natl. Acad.Sci.USA.* 50:425.

Witkin, E.M. 1967. The radiation sensitivity of *E.coli* B: a hypothesis relating filament formation and prophage induction. *Proc.Natl. Acad.Sci.USA.* 57:1275.

Witkin, E.M. 1976. Ultraviolet mutagenesis and inducible DNA repair in *E.coli*. *Bacteriol.Rev.* p.869.

Witkin, E.M. & Kirschmeier, P. 1978. Complexity in the regulation of SOS function in bacteria. In *DNA Repair Mechanisms* (eds P.C. Hanawalt, E.C. Friedberg & C. Fox), p.371. New York, San Fransisco & London: Academic Press.

Woo, D.D.L., Holt, S.C. & Leadbetter, E.R. 1978. Ultrastructural examination of all surface features of *Bacteroides melaninogenicus* subspecies. *J.Dent.Res.* 57:215.

Woo, D.D.L., Holt, S.C. & Leadbetter, E.R. 1979. Ultrastructure of *Bacteroides* species: *Bacteroides asaccharolyticus, Bacteroides fragilis, Bacteroides melaninogenicus* subspecies *melaninogenicus*, and *B.melaninogenicus* subspecies *intermedius*. *J.Infec.Dis.* 139:534.

Woods, D.R. & Thompson, J.A. 1975. Unstable generalized transduction in *Achromobacter*. *J.Gen.Microbiol.* 88:86.

Wüst, J. & Wilkins, T.D. 1978. Effect of clavulanic acid on anaerobic bacteria resistant to beta-lactam antibiotics. *Antimicrob.Agents. Chemother.* 13:130.

Yamamoto, N. 1967. Recombination: damage and repair of bacteriophage genome. *Biochem.Biophys.Res.Commun.* 27:263.

Yasuda, S. & Sekiguchi, M. 1970. T4 endonuclease involved in repair of DNA. *Proc.Natl.Acad.Sci.USA.* 67:1839.

Youngs, D.A. & Smith, K.C. 1973a. Evidence for the control by *exr* A and *pol* A genes of two branches of the *uvr* gene-dependent excision repair pathway in *Escherichia coli* K-12. *J.Bacteriol.* 116:175.

Youngs, D.A. & Smith, K.C. 1973b. Involvement of DNA polymerase III in excision repair after ultraviolet irradiation. *Nature. New Biol.* 244:240.

Youngs, D.A. & Smith, K.C. 1977. The involvement of polynucleotide ligage in repair of UV induced DNA damage in *E.coli* K-12 cells. *Mol.Gen. Genet.* 152:37.

Zehnbauer, B. & Markovitz, A. 1978. Cloning of a gene that controls radiation sensitivity, cell division, and capsular polysaccharide synthesis, the *lon (cap* R*)* gene. In *DNA Repair Mechanisms* (eds P.C. Hanawalt, E.C. Friedberg, & Fox, C.F.), Vol. IX, p.797. New York: Academic Press.

Zelle, M.R. & Hollaender, A. 1954. Monochromatic ultraviolet action spectra and quantum yields for inactivation of T1 and T2 *E.coli* bacteriophages. 68:210.

Zetterberg, G. 1964. Mutagenic effects of UV and visible light. In *Photophysiology* (ed. A.C. Giese), Vol. 3, p.247. New York: Academic Press.

Zieg, J., Silverman, M., Hilmen, M. & Simon, M. 1977. Recombinational switch for gene expression. *Science* 196:170.

Zinder, N.D. & Lederberg, J. 1952. Genetic exchange in *Salmonella*. *J.Bacteriol.* 64:679.