

CHARACTERIZATION AND MODE OF ACTION OF
A BACTERIOCIN PRODUCED BY A
BACTEROIDES FRAGILIS STRAIN

BY

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SUMMARY

Bacteroides fragilis strain Bf-1 produces an extracellular bacteriocin at the beginning of the stationary growth phase. Production is not inducible by either ultraviolet light or mitomycin C. The low molecular weight bacteriocin (MW estimates of 13 500 and 18 800 obtained from Sephadex G-100 chromatography and SDS-PAGE electrophoresis respectively) is stable between pH 7 - 9 and is inactivated on incubation with trypsin and pronase. An unusual feature of the Bf-1 bacteriocin is its apparent biphasic temperature stability: while the majority of the activity (97%) is destroyed by heating at 60°C ($t_{\frac{1}{2}} = 2.5$ min at 60°C), a small proportion (3%) is stable even after autoclaving at 121°C for 15 min.

The killing of sensitive cells occurs in 2 stages and the killing action is reversed by incubation with trypsin. The transition from stage I to stage II is dependent on the temperature of incubation and the growth state of sensitive cells. 2,4-Dinitrophenol prevents this transition. The Bf-1 bacteriocin has an unusual mode of action. It specifically inhibits RNA synthesis whilst having no effect on protein or DNA synthesis. No effect on intracellular ATP levels were observed. The heat-stable (3%) fraction had a similar biochemical effect. In vitro studies involving RNA polymerase indicated that the bacteriocin and the antibiotic rifampicin have similar effects on RNA synthesis. The bacteriocinogenic strain (Bf-1) is insensitive to its own bacteriocin both in vivo and in vitro, although this immunity is overcome in vitro by the addition of higher concentrations of the Bf-1 bacteriocin.

The bacteriocinogenic strain (Bf-1) harbors a cryptic plasmid (or plasmids) which on a neutral sucrose gradient, sediments faster than the Col E1 marker plasmid DNA. Attempts to cure this strain of its bacteriocinogenic phenotype were unsuccessful.

INTRODUCTION .

The existence of anaerobic bacteria was first recognized by Pasteur who noticed that some micro-organisms can live in the absence of oxygen, a gas previously considered essential for the maintenance of all life. He introduced the terms "aerobic" and "anaerobic" to designate, respectively, life in the presence and absence of air. Since then, a great diversity of bacterial genera have been reported which can also live in the absence of oxygen.

Stanier, Doudorff & Edelberg (1971) distinguished between an obligate anaerobe and a facultative anaerobe, where obligate anaerobes are organisms which are killed or their growth inhibited by molecular oxygen, and facultative anaerobes as those which can grow either in the presence or absence of oxygen. Morris (1976) described an obligate anaerobe as an anoxybiontic, aero-intolerant organism, that is, an organism which (1) is capable of generating energy and synthesizing its substance without recourse to molecular oxygen, and (2) demonstrates a singular degree of adverse sensitivity to oxygen which renders it incapable of growing in air at 1 atmosphere.

From his findings with a selection of anaerobes, Loesche (1969) concluded that at least three subgroups were identifiable, viz.:

1. Strict anaerobes which would grow on spread plates only when the atmosphere contained less than 0.5% O_2 , e.g. Treponema denticola, Selenomonas ruminatum, Clostridium haemolyticum.
2. Moderate anaerobes which would generally grow on plates when the atmosphere contained less than 8% O_2 , e.g.

Bacteroides fragilis, Clostridium novyii Type A,
Peptostreptococcus elsderii.

3. Microaerophiles which require a low concentration of O_2 for optimal growth but are still unable to grow in air e.g. Vibrio fetus.

The fact that anaerobes differ so widely in their sensitivity to molecular oxygen has led to the hypothesis that the action of oxygen on anaerobic bacteria occurs in two phases. The first phase is bacteriostatic and the organism is thought to protect itself by use of its internal reducing power. The only ill effects of aeration in this phase would be a suppression of metabolism because of the diversion of reducing power in order to detoxify the oxygen. The cessation of butyrate production in favour of acetate formation in aerated cultures of saccharolytic Clostridium spp (O'Brein & Morris, 1971), and the decrease in glucose consumption and volatile fatty acid production in an aerated continuous culture of Bacteroides fragilis subsp. fragilis (Onderdonk, Johnston, Mayhew & Gorbach, 1976), provide evidence for the diversion of reducing power. The abrupt decrease in intracellular ATP content in aerated cultures of Clostridium acetobutylicum with the equally abrupt rise in ATP levels when anaerobic conditions are restored, illustrates the rapidity of these events (O'Brein & Morris, 1971).

Some anaerobes, are, therefore, able to sustain their viability under aerobic conditions by reducing their surroundings at the cost of depressing their metabolic functions. This phenomenon has its counterpart in some aerobic bacteria which divert reducing power to scavenge molecular oxygen so as to shield key oxygen-labile components from direct contact with oxygen (Jones, Brice, Wright & Ackrell, 1973). The nitrogenase activity of Azobacter vinelandii was inhibited by molecular oxygen. This inhibition

was reversible with mild oxygenation but irreversible inhibition occurred with vigorous oxygenation. Increased respiration was thought to scavenge the oxygen and hence protect the oxygen-sensitive sites of the enzyme but vigorous aeration appeared to overcome these respiratory functions and led to irreversible inhibition of the enzyme. Presumably this defensive mechanism is not available to those strict anaerobes which have no 'spare' reducing power which they may divert to these ends, and moderate anaerobes might have this to a greater or lesser extent which would explain the difference in observed sensitivity to oxygen.

Phase 2, the bactericidal phase, occurs when the reductive Phase 1 defenses have been overwhelmed by continued, excessive oxygen (Morris, 1976). Irreversible damage to the cell then occurs. Among the hypotheses proposed to explain the oxygen intolerance of obligate anaerobes are the following (Morris, 1976):

(a) Oxygen is toxic:

- (i) Presence of free oxygen in the culture medium is incompatible with the attainment and maintenance of the low culture Eh value required for growth.
- (ii) Preferential reduction of exogenous oxygen drains the organism of reducing power which it requires for biosynthetic processes.
- (iii) The anaerobe is particularly vulnerable to oxygen because its metabolism and growth depends on key components liable to 'autotoxidation' (SH compounds, iron sulfur proteins, flavoproteins and tetrahydropteridines are examples).

(b) Oxygen itself is not toxic:

- (i) Reducing agents in nutrient media became toxic on exposure to oxygen. The nature of these inhibitory substances has not yet been clarified, but organic peroxides, aldehydes and free radicals have been implicated.
- (ii) Toxic products are invariably formed by the interaction of oxygen with reduced cell constituents, as well as by the action of certain oxidases and flavin dehydrogenases. Examples of these toxic products are hydrogen peroxide, superoxide anion, hydroxyl radical and singlet oxygen.

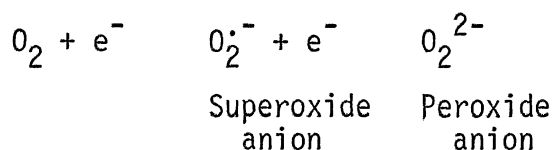
It was initially thought that oxygen exerts its toxic effect by elevation of the culture Eh (Smith & Holdeman, 1968). However, O'Brein & Morris (1971), demonstrated normal growth of Clostridium acetobutylicum in media where the Eh was maintained at +370 MV by the addition of ferricyanide though not at +100 MV when this Eh was produced by aeration. Furthermore, aeration of ferricyanide maintained culture then halted growth even though there was no further increase in culture Eh (O'Brein & Morris, 1971). Similar results were obtained by Onderdonk et al. (1976) when they observed that alterations of Eh alone by chemical means did not influence growth of Bacteroides fragilis within a fermentor. Most investigators therefore felt that a change in Eh alone could not be the cause of oxygen lethality in obligate anaerobes and the theory of toxic products or by-products of oxygen metabolism in anaerobes has recently received much attention.

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It was initially thought that anaerobes were aerò-intolerant because they lacked the enzymes catalase or peroxidase and as such could not detoxify the hydrogen peroxide which was formed during the course of oxygen metabolism. Because hydrogen peroxide is a highly reactive compound, this was seen to be a form of "microbial" suicide. Subsequently, Holdeman & Moore (1972) showed that large numbers of obligate anaerobes produced this enzyme. Gregory, Vetri, Wagner & Wilkins (1977) discovered that catalase production in Bacteroides fragilis, B. distasonis and B. thetaiotaomicron was suppressed by the presence of glucose or any other carbohydrate. Since anaerobes are routinely cultivated in media containing a carbohydrate, this would explain why this enzyme was not detected initially. Furthermore, Gregory, Kowalski & Holdeman (1977) reported the requirement of exogenously supplied hemin for the detection of catalase activity and under these conditions, the level of catalase activity of the anaerobe B. distasonis was found to be similar to that of the aerobes Escherichia coli and Streptococcus faecalis.

One product of oxygen metabolism which has lately achieved much prominence is the superoxide anion $O_2^{\cdot -}$. This is a highly reactive radical and is formed by the univalent reduction of molecular oxygen.



In aqueous solutions this radical is synthesized by the interaction of molecular oxygen with various cellular constituents including reduced flavin, flavoproteins, quinones, iron-sulfur proteins and tetrahydropteridines. It is also formed by the action of certain enzymes including aldehyde oxidase and numerous flavin dehydrogenases (Morris, 1976). It can act as a potent oxidizing or reducing agent and has the additional

hazardous potential of producing three more derivatives of oxygen, viz. hydrogen peroxide, hydroxyl radical and singlet oxygen. McCord & Fridovich (1969) first discovered the superoxide dismutases group of enzymes. These enzymes were found to be lacking in a number of strictly anaerobic bacteria but were present in aero-tolerant organisms, both catalase-positive and catalase-negative (McCord, Keele & Fridovich, 1971). The fact that superoxide dismutase is a scavenger of the potentially detrimental free-radical form of oxygen (O_2^-), McCord, Keele & Fridovich (1971) proposed the Superoxide Dismutase Theory of Anaerobiosis which states that it is their total lack of superoxide dismutase that is the cause of the aero-intolerance of obligate anaerobes. This theory was further supported by Costilow & Keele (1971) who reported oxygen tolerance in Bacillus papilliae was more dependent on superoxide dismutase than on catalase. Gregory, Yost & Fridovich (1973) showed Escherichia coli B contains two superoxide dismutases; one Fe-containing which was located in the periplasmic space, and the other, manganoprotein, found inside the cell. Independent manipulations of the levels of these enzymes showed that cells rich in the periplasmic enzyme were particularly resistant to the bactericidal action of exogenously generated superoxide anions, whilst only those rich in the cytoplasmic Mn-enzyme showed enhanced resistance to hyperbaric oxygen and to streptonigrin (an antibiotic whose lethality is markedly enhanced by O_2 and whose action has been supposed to involve the generation of O_2^- (Gregory, Yost & Fridovich, 1973).

Some superoxide dismutase activity has been found in various aero-intolerant bacterial species including Chlorobium, Desulfovibrio and Clostridium spp. (Morris, 1976), and B. distasonis (Gregory et al., 1977). These enzymes could not be induced by increased oxygen levels. What can be concluded so far about this enzyme is that it is required by all aerobic

organisms as a defense against the by-products of oxygen metabolism and that some strict anaerobes do possess a low level of the enzyme. Whether this enzyme affords anaerobic bacteria greater tolerance towards oxygen is still not yet established.

Anaerobic bacteria are now recognized as the predominant flora in the intestinal flora of man (Finegold, 1969; Finegold, 1970; Moore, Cato & Holdeman, 1969). The most prevalent anaerobe is Bacteroides fragilis with an average count of 10^{10} to 10^{11} per gram and Moore et al. (1969) identified 46% of the 230 strains of faecal bacteria isolated from 2 people as this species. However, at least 36 other species were isolated from the same specimens. Other anaerobic Gram-negative bacilli such as Sphaerophorus species and Fusobacterium spp were also abundant (Draser, 1967; Dubos et al., 1963; Finegold, 1969; Finegold, 1970; Moore et al., 1969). Propionibacterium, Peptostreptococcus and Clostridium species were also found in varying numbers. Little work has been done on the actual sites in which these bacteria are found in vivo in humans, but Savage & Dubos (1967) have demonstrated that bacteria and yeasts occupy specific microenvironments in the mouse intestine. These layers of organisms were formed early in life and persisted throughout the life of the animal, provided nutrition was undisturbed.

Little is known about the significance of these organisms in the alimentary tract of animals and humans. Savage & Dubos (1967) suggested that the anaerobes might play an essential role in maintaining the integrity of the water transport mechanism in the intestinal epithelium in mice. Although ruminants are dependent on bacterial action for the utilization of their normal diet (Bryant & Burkey, 1953) the extent of contribution of these bacterial products to nutrition is uncertain. In humans they appear to be involved in the defense against infection and various aspects of host

nutrition. They are capable of synthesizing numerous vitamins including B₁, B₂, B₆, B₁₂, K, folic acid, and of deconjugating bile acids (Drasar & Hill, 1974; Finegold, 1969; Finegold, 1970). The normal flora is also becoming increasingly implicated in cancer of the large intestine, by virtue of their ability to metabolise a procarcinogen to produce carcinogens such as nitrosamines from nitrates. They also produce substances known to be carcinogenic from bile acids and cholesterol (Drasar & Hill, 1974; Wilkins & Hackman, 1974). Salyers et al. (1977) reported on the ability of Bacteroides spp. especially B. thetaiotaomicron to ferment mucin and plant polysaccharides. This may have significance in disease such as colon cancer, as mucin degradation impairs the protective layer lining the intestine and increases its vulnerability to harmful substances (Burt, 1977b).

Detailed knowledge of the normal bowel flora is essential since they have been implicated in a number of infections such as appendiceal abscess, peritonitis, pneumonia, subphrenic and subhepatic abscess, pyogenic liver abscess, pylephlebitis, diverticulitis, peri-rectal abscess and postoperative infections following surgical operations in the bowel or elsewhere in the abdomen. Gram-negative anaerobic bacilli, particularly Bacteroides species are often associated with such infections and can be isolated in pure culture or as mixed infections with other anaerobes or facultative organisms (Dubos et al., 1963; Felner & Dowell, 1971; Finegold, 1969; Finegold, 1970; Sutter et al., 1975).

The unique ecological niche of Bacteroides, its particularly important role in human physiology and its importance as an opportunistic pathogen have made this genus the subject of much current investigation. Since the knowledge of the genetics of this genus is virtually non-existent (Morris, 1975), initial studies in this field have been directed on the isolation and characterization of plasmids harboured by Bacteroides strains with the aim of establishing their phenotypes (Tinnel & Macrina, 1976).

Plasmids are stable, extrachromosomal genetic elements which control a variety of bacterial properties including conjugation (Novick, 1969; Clowes, 1972), resistance to antibodies and heavy metals (Helinski, 1973; Holloway et al., 1971) and bacteriocins (Clowes, 1972; Hardy, 1975; Nomura, 1967). Plasmids also control the production of exfoliative toxins in S. aureus (Warren et al., 1975), hydrocarbon metabolism (Chakrabarty et al., 1973) and surface antigens such as the K88 antigen of E. coli (Stirm et al., 1967; Bak et al., 1972).

Many of these plasmids confer pathogenicity on their host strain, for example, the production of K88 antigen in E. coli is linked to the ability to cause disease in piglets (Stirm et al., 1967). Furthermore, it has recently been shown that the presence of the Col V factor in E. coli strains was responsible for a number of deaths produced by E. coli in chickens, guinea pigs and rabbits (Smith, 1974). Other examples of plasmid conferred pathogenicity on host strains are exfoliative toxin production by S. aureus (Warren et al., 1975) which is responsible for certain skin diseases and the pathogenicity of Agromobacter tumiefaciens (Van Larebeke et al., 1974; Zaenen et al., 1974).

The pathogenic nature of Bacteroides strains (Finegold, 1969; Finegold, 1970; Smith & Holdeman, 1968) and their resistance to a large variety of antibiotics (Finegold & Sutter, 1971; Kislak, 1972; Martin et al., 1972; Sutter & Finegold, 1976; Thornton & Cramer, 1971) has led to speculations that these properties are plasmid coded. These speculations have been strengthened by the fact that strains in this genus have been found to produce bacteriocins (Beerens & Baron, 1965; Beerens et al., 1966; Booth et al., 1977; Podhaisky & Reinhold, 1970). Subsequently, studies on the occurrence of plasmids within strains of this genus were undertaken to determine whether any of these traits were plasmid-controlled.

The first report on the presence of plasmids within these strains was published by Stiffler et al. (1974) who screened four strains of clinical isolates of B. fragilis ss. fragilis. Two of these strains contained plasmids of molecular weight ca. 4×10^6 which were present in multiple copies per cell. The third strain contained two plasmid types: a 2.7×10^6 plasmid present in multiple copies and a second 16×10^6 molecular weight plasmid, present in only a few copies per chromosomal equivalent. The fourth strain contained no detectable plasmid. Damle & Syed (1975) later reported the presence of plasmids in two oral Bacteroides strains, a clinical isolate of B. ochraceus which contained a 17×10^6 MW plasmid and a 5×10^6 MW plasmid and a B. melaninogenicus strain containing a 17×10^6 MW plasmid. In 1975 Guiney & Davis showed that two clinical isolates of Bacteroides contained covalently closed circular DNA by sedimentation through an alkaline sucrose density gradient. The B. fragilis strain was found to contain a homogeneous species of plasmid, having a molecular weight of ca. 25×10^6 while the B. ochraceus strain contained two distinctly different plasmids, the larger having a MW of 70×10^6 , and the smaller a MW of 25×10^6 . While the B. fragilis strain was resistant to tetracycline, the B. ochraceus strain was found to be multiply-resistant to a number of antibiotics. In a recent publication, the molecular weights of the B. ochraceus plasmids were revised as 70×10^6 (plasmid designated pG10) and 37×10^6 (plasmid designated as pGD11) (Guiney & Davis, 1978). Furthermore, Guiney & Davis (1978) reported the first positive identification of a plasmid harboured by a Bacteroides strain. The pG10 plasmid was identified as a conjugative plasmid, using an E. coli recipient and it specifies resistance to tetracycline, chloramphenicol, kanamycin and streptomycin. The pG10 can thus be transferred by conjugation to an E. coli recipient and is stably maintained in the recipient. Tinnell and Macrina (1976) screened strains

from seven different DNA homology groups of B. fragilis and from three different groups of B. thetaiotaomicron. Six of the strains contained no detectable plasmid DNA following caesium chloride-ethidium bromide equilibrium centrifugation, but the other four strains had satellite bands corresponding to the CCC plasmid DNA. Two B. fragilis ss fragilis strains had plasmids of molecular weights 31×10^6 and 3×10^6 . In all instances, the small molecular weight plasmids were present in multiple copies per cell while the larger plasmids were only present in a few copies per chromosomal equivalent. The biological function of these plasmids and the significance of plasmid copy number is unknown at this stage.

The multiple antibiotic resistance demonstrated by Bacteroides strains isolated led to the hypothesis that these strains may act as reservoirs of antibiotic resistance genes (Guiney & Davis, 1978) and that R plasmids could be transferred, in vivo, from e.g. an E. coli donor to a Bacteroides recipient in the gastro-intestinal tract. Since the environment in the mammalian gastro-intestinal tract is mainly anaerobic (Askevold, 1956) such a transfer has to occur under anaerobic conditions. Although R plasmid DNA transfer has been demonstrated in E. coli under aerobic conditions, it was initially thought that chromosomal transfer by an E. coli Hfr strain was inhibited under anaerobic conditions (Fisher, 1957). This theory was strengthened by Mitsuhashi (1965) who showed that anaerobiosis completely inhibited R plasmid transfer from a Shigella flexneri donor to an E. coli recipient. However, Stallions & Curtiss (1972) showed that chromosome transfer in E. coli does occur at high frequencies under anaerobic conditions and Moodie & Woods (1973a) reported an R plasmid transfer in E. coli under anaerobic conditions. This contradiction was explained by Burman (1975) who reported that the transfer of certain R plasmids was unaffected by anaerobiosis while that of others was depressed.

Attempts were therefore made to demonstrate in vitro resistance transfer between strains of E. coli and strains of the Bacteroides group. Although initial attempts proved unsuccessful (Anderson & Sykes, 1973; Guiney & Davis, 1975; Del Bené et al., 1976) antibiotic resistance transfer has been demonstrated to obligate anaerobes from E. coli (Burt & Woods, 1976). This was only possible after heat-treatment of the recipients, which presumably inhibits restriction systems of the anaerobes, as in E. coli (Burt & Woods, 1976); Cohen et al., 1972). Manchini & Behme (1977) also reported the successful multiple antibiotic resistance transfer from B. fragilis to an E. coli recipient. However, in this case the resistance markers were found to be unstable in the E. coli transconjugants and were found to segregate at a high frequency and furthermore, supercoiled DNA could not be detected in either the B. fragilis donor or the E. coli recipient. Guiney & Davis (1978) showed R plasmid transfer between a B. ochraceus donor and a restriction deficient E. coli recipient. The difficulties encountered in demonstrating R plasmid transfer, could mean on the other hand, that Bacteroides strains are perhaps not acting as reservoirs of antibiotic resistance genes in the gastro-intestinal tract. Very little work has been done on phages of Bacteroides strains. Sabiston & Cohl (1969) isolated the first B. distasonis phage and Brandis et al. (1972) described the isolation of B. fragilis phages. These phages were found to lyse 23 out of 68 B. fragilis strains tested, but could not infect other Bacteroides strains. Keller & Traub (1974) characterized a B. fragilis phage which could establish a phage carrier state with the host bacterium. The only report of a temperate phage in this group of anaerobes was made by Bradley (1972) who studied the ultra-structural changes in infected Sphaerophorus varius cells. Burt (Ph.D. Thesis, 1977) described the isolation of four phages which could infect B. thetaiotaomicron. These phages were designated β_1 , β_2 , β_3 and β_4 . One of the phages, β_1 , was further characterized and found to produce

defective particles at a high frequency. Burt and Woods (1977) reported on the first successful transfection of B. thetaiotaomicron with phage DNA. This is the first report of phage DNA uptake in a Bacteroides strain and since spontaneous genetic transfer has not been described in Bacteroides species (Burt & Woods, 1977), the transfection procedure may be utilized to develop a transformation system.

The most extensively studied bacteriocinogenic factors are those of E. coli, termed colicinogenic (Col) factors (Hager, 1973; Hardy, 1975; Reeves, 1972). These genetic determinants of colicin production exist in bacterial cells as stable extrachromosomal elements (plasmids). The Col factors can be divided into two major groups according to their ability to transfer themselves. Only the larger plasmids (Col V and Col Ib) are self-transmissible and are designated sex factors (Helinski, 1973) since they are capable of promoting their own transfer or the transfer of the host chromosome to the recipient cell. The Col factors are generally regarded to exist in the cell in the form of covalently closed circular DNA molecules. They can recombine with other plasmids as illustrated by the Col V. Col B.trp.cys. plasmid, a recombinant of Col and F factors isolated by Fredericq (1969).

After purification most bacteriocins studied appear to be proteins (Hager, 1973; Hardy, 1975; Reeves, 1972; Tagg et al., 1976) but others are complex molecules consisting of protein, carbohydrate and lipid (Tagg et al. 1976). Of interest are the colicins A, V, K and SG 710 which are lipopolysaccharide-protein complexes, identical to, and possessing the serological specificity of the producer strain (Reeves, 1972). Jesaitis (1970) who found that colicin K, isolated from a strain of Proteus mirabilis after induction with mitomycin C, was a protein, free of lipopolysaccharide, suggested that colicins released spontaneously might be lipopolysaccharide-protein complexes and that on induction too much colicin was synthesized

to associate with the O antigen complex and the excess colicin as such, remained as a protein.

The essential characteristic of bacteriocins, which led to the discovery of colicin V by Gratia in 1925, is the striking similarity between the phenomena of bacteriocinogeny and lysogeny (Hardy, 1975; Mayr-Harting et al., 1972; Reeves, 1972). Among the similarities in the two phenomena are:

- (i) Bacteriocins and bacteriophages adsorb to specific receptors on the same surface, and in some cases, share the same receptor site (Nomura, 1967). In some notable cases, some bacteriophages and bacteriophage "ghosts" have similar effects on a sensitive cell as bacteriocins (Kaziro & Tanaka, 1965; Nomura, 1967).
- (ii) The genetic determinants of bacteriocins and bacteriophages have been classed as episomal (Jacob & Wollman, 1958), and perhaps prophages which do not have a specific locus on the bacterial chromosome, e.g. prophage P1 (Ikeda & Tomizawa, 1968) may be intermediate between the majority of the bacteriocinogenic factors which cannot recombine with the chromosome and temperate phages.
- (iii) Certain bacteriocins and bacteriophages are induced by inhibitors of DNA synthesis. Moreover, lacunae obtained after UV irradiation increase in size or numbers after UV treatment. A similar phenomenon is observed when lysogenic bacteria are treated with UV.

- (iv) The immunity which is determined by Col factors against colicins is similar to the immunity conferred by bacteriophage genomes against defective bacteriophages (bacteriophage "ghosts") (Hardy, 1975).

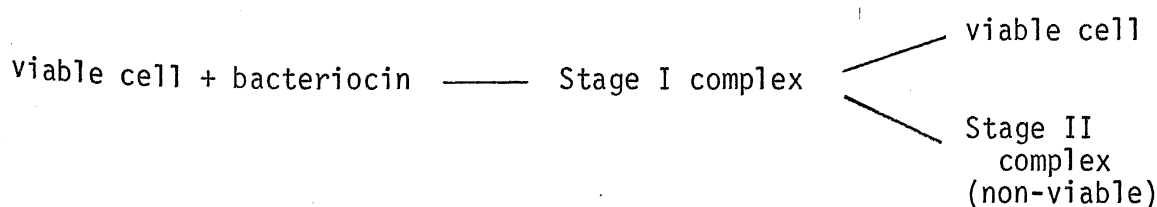
Although no evidence for this exists at this stage, these similarities have led certain investigators (Hardy, 1975; Reeves, 1972) to speculate on the possible evolutionary relationship between bacteriocins and bacteriophages. Fredericq (1963) in fact suggested that bacteriocinogenic factors and phage DNA may have undergone recombination, resulting in the incorporation of bacteriocin genes into the phage genome, which might account for bacteriocin-like effects on sensitive-like cells (Reeves, 1972).

Perhaps the aspect of bacteriocin study which has aroused most interest are their modes of action. The discovery of the first antibacterial element (colicin V) by Gratia (1925) caused a minor flurry of excitement which was followed by a low background noise level for almost 40 years. Even the discovery that colicins could kill sensitive cells by a single-hit mechanism and that the lethal action was reversed by incubation with trypsin did not raise any great interest in the study of bacteriocins. It was only after Nomura and his co-workers (Bowman et al., 1971; Almendinger & Hager, 1971; Nomura, 1963; Nomura, 1967) had established that different colicins had distinct biochemical effects that the coming of age in bacteriocin research was reached.

To date, two stages in bacteriocin action are defined: The first stage occurs when the bacteriocin molecule comes into contact with the sensitive cell but has not yet initiated a series of events leading irreversibly to cell death. It has been established (Fredericq, 1946; Mayr-Harting & Shimeld, 1965; Maeda & Nomura, 1966; Reeves, 1965) that colicins bind to

the surface of sensitive cells. Further studies revealed that there were specific receptors to which the bacteriocins would bind (Reeves, 1972; Sabet & Schnaitman, 1973; Braun et al., 1973). Cross-resistance studies revealed that there were common receptors for certain bacteriophages and colicins (Fredericq, 1949; Fredericq & Smārda, 1970) and for different colicins (Nomura, 1967). The receptor for colicin M and phage T5 has been isolated and purified and was found to be a single peptide of molecular weight 85 000 (Braun, Schaller & Wolff, 1973).

Not much is known about the cellular location of these receptors. While some evidence points to a cell envelope location, especially studies with radioactively labelled colicins (Maeda & Nomura, 1966) other studies strongly suggest a cytoplasmic membrane location (Bhattacharyya et al., 1970; Smarda & Taubeneck, 1968). Moreover, the fact that some lethal effects of colicins can be reversed by incubation with trypsin does not help to substantiate any of these two schools of thought since it would be possible for colicin molecules to attach to receptor sites on the cytoplasmic membrane through bridges in the cell wall. Perhaps, at this stage it would be plausible to accept these two theories by assuming that different bacteriocins have different physico-chemical properties and would, in effect, bind differently to different receptors. Cells which are still in stage I can still be rescued from bacteriocin action by incubation with trypsin or chymotrypsin (Nomura & Maeda, 1965; Nomura & Nakamura, 1962; Reynolds & Reeves, 1963). Luria (1964) and Nomura (1964) independently suggested similar models on the mechanism of bacteriocin action. Although Nomura's model (Hager, 1973) places emphasis on the cell membrane, the basic feature of the two models are similar. A summary of the two models is as follows:



Studies carried out at different laboratories indicate that the transition from stage I to stage II is dependent both on the temperature of incubation and the physiological stage of the sensitive cell (Tagg *et al.*, 1976; Mahony & Butler, 1971; Elgat & Ben-Gurion, 1969). Jetten & Vogels (1974) in fact reported that cells pregrown at 37°C were more sensitive to staphylococin 1580 than were cells pregrown at 20°C. The stage which brings the bacteriocins into effective contact with the target of their biochemical action may be the energy-dependent step which is inhibited by uncouplers (Hardy, 1975; Plate & Luria, 1972; Jetten & Jetten, 1975). The energised state of the cytoplasmic membrane is necessary for the transition from stage I to stage II (Jetten & Jetten, 1975).

Maeda & Nomura's (1966) observations with fractionated cells after 60 min contact with radioactively labelled colicin E2-P9 prompted them to suggest a mechanism whereby colicins attached to receptors exert their effects on sensitive cells. According to this model, each colicin has a "killing" or "biochemical" target, or both, and that the colicin attached to the receptor site exerts its effect by a specific stimulus sent through a specific transmission system, presumably located in the cytoplasmic membrane. Almendinger & Hager (1972) supported Nomura's model by proposing a hypothesis for colicin E2 through displacement of endonuclease I in the periplasmic space to the cytoplasm. This theory was further strengthened by the fact that *E. coli* mutants which are deficient in endonuclease I were resistant to colicin E2 action (Almendinger & Hager, 1972). The model suggested by Nomura emphasised the functional importance of the cellular membrane. It was further suggested (Luria, 1964; Nomura, 1964; Nomura & Maeda, 1965; Maeda & Nomura, 1966) that all the biochemical and killing targets of known colicins

and other bacteriocins studied, have a physical connection to the cell membrane, or are part of it. The DNA in bacterial cells (an apparent target for colicin E2) or ribosomes (target for colicin E3) may have physical connections to the cell membrane (Nomura, 1967). Furthermore, the inhibition of bacteriocin action by uncouplers of oxidative phosphorylation could be easily explained since the oxidative phosphorylation system resides in the membrane. Nomura (1967) thus suggested that the bacterial cell membrane consists of several parts, each of which may be individually affected by stimuli initiated by specific colicins at receptor sites.

This theory became doubtful, however, when Boon (1971) and Bowman et al., 1971) discovered that colicin E3 may act directly as a degradative enzyme on ribosomal RNA. Boon (1971) & Bowman et al. (1971) showed that incubation of the ribosomes prepared from 30S extracts with purified colicin E3 resulted in their inactivation. The 50S extracts were not affected. The 16S RNA, from the 30S extract, was found to lose a terminal fragment (about 7 nucleotides from the 3'-OH end), similar in size to the fragment released upon in vivo treatment with colicin E3. Crude extracts (containing ribosomes) derived from E3-colicinogenic cells were found to be resistant to colicin E3 in vitro. However, incubation of ribosomes purified from these extracts with purified colicin E3 resulted in cleavage (Bowman et al., 1971). Thus, it is apparent that the presence of the Col factor not only confers the ability to produce a particular colicin, but also the ability to produce a substance which would inhibit colicin action in vivo and in vitro (Bowman et al., 1971). Such an inhibitor has been purified by Nomura's group (Hager, 1973) and is primarily a protein and very acidic.

Jakes and Zinder (1974) reported that highly purified colicin E3 contains an immunity protein. He attempted to explain the transport of colicins from producer cells to susceptible organisms by postulating that the

immunity protein had a dual fraction: to protect the colicin molecule during the transport process and to bind to receptors on susceptible cells. This protein would remain attached to the cell surface when the colicin molecule or part of it enters the cell. This protective function towards the "active site" of the bacteriocin has been confirmed by limited trypsinolysis of cloacin DF13 (Mooi & de Graaf, 1976). However, recently Schaller & Nomura (1976) purified colicin E2 from its immunity protein. They showed that colicin E2^x (freed from its immunity protein) can bind to cell receptors and concluded that the immunity protein was not required for binding to receptors. Moreover, they found that colicin E2^x had DNA endonuclease activity which makes the earlier hypothesis of Almendinger & Hager (1971) doubtful. There have been relatively few reports of the nature of the morphological changes produced in susceptible bacteria on treatment with bacteriocins. Clawson & Dajani (1970) showed that extensive structural changes occurred in a group A streptococcus strain after exposure to staphylococcin C55 (Tagg *et al.*, 1976). Morphological changes observed included condensation of nuclear material partial loss of ribosomes, modification of mesosomes, and eventual dissolution of cell contents. No effect on the cell wall was noticed. Similar changes were observed in sensitive bacteria treated with streptococcin A-FF22 (Tagg *et al.*, 1973) and boticin E-S51 (Ellison *et al.*, 1971).

Gratia's discovery that E. coli V, which produced "principle V" (later known as colicin V) caused virulence in rabbits and guinea pigs adds interest to the recent findings that increased invasiveness of E. coli strains in chickens is strongly associated with the Col V⁺ character (Williams Smith, 1974). Although other Col factors, such as Col E and Col I factors, did not increase the number of deaths in chickens injected with bacteria carrying these plasmids, William Smith's discovery suggested

that the bacteriocinogenic strains could play a role of pathological importance.

The importance of Bacteroides strains and their lack of a genetic system have been the reasons for our current interest in their study. Moreover, since most bacteriocins are produced by plasmids (Nomura, 1967; Reeves, 1972), the establishment of a plasmid borne phenotype for Bacteroides fragilis involving "curing" and transformation was the initial aim of the research presented in this thesis. Furthermore, it was also considered important to characterize and study the mode of action of a Bacteroides bacteriocin as this has not been done previously.

CHAPTER II

ISOLATION AND DETECTION OF BACTERIOGINOGENIC STRAINS

Thirty-six Bacteroides strains were isolated. Of the 36, 20 were found to exhibit bacteriocin-like antagonism against at least one of the strains tested. Two strains, a producer strain Bf-1 and a sensitive indicator strain Bf-2 were selected for further study.

2.1 INTRODUCTION

Not much work has been done on obligate anaerobic bacteria, mainly because of the difficulties encountered in their isolation, transportation and subsequent cultivation. Many bowel anaerobes are extremely fastidious and require stringent conditions for isolation and culture. The roll tube method developed by Hungate (1969) for obligate rumen anaerobes made it possible to eliminate exposure to oxygen during the entire isolation and cultivation procedure. To avoid the formation of peroxides and other organic inhibitors, methods of preparing pre-reduced and anaerobically sterilized (PRAS) media were also developed. Such techniques, whereby low Eh values (-150 mV or lower) can be achieved, have been used to isolate obligate anaerobes from the intestinal contents of various animals (Holdeman & Moore, 1972) and the mouth (Gordon et al., 1972) and intestine (Holdeman & Moore, 1972; McMinn & Crawford, 1970) of humans.

This roll tube method is difficult and cumbersome and not suitable for techniques involving agar plates. As an alternative better method for obtaining anaerobic conditions, anaerobic glove cabinets have been developed (Aranki et al., 1969; Leach et al., 1971; Lee et al., 1968; Moodie & Woods, 1973b; Roseblatt et al., 1973). Methods of varying complexity were used to remove oxygen from these cabinets. One technique (Lee et al., 1968)

involved repeated inflation with nitrogen from a balloon inside the box. The displaced air was then replaced with a 95% N₂ - 5% CO₂ gas mixture. This method was not very satisfactory, since the methylene indicator (Eh = 11 mV at pH 7.0, Smith & Holdeman, 1968) did not always become completely reduced. The anaerobic glove cabinet constructed by Moodie & Woods (1973b) was flushed for at least 3 hours with a CO₂-H₂ mixture which was passed through a Deoxo catalyst which removed traces of oxygen. Any remaining oxygen was removed by palladium catalyst pellets inside the box. This method has proved satisfactory for the cultivation and isolation of anaerobes and has been employed for that purpose in this laboratory.

Although the detection and isolation of bacteriocins presents a few problems, a number of bacteriocin-producing strains have been isolated. The main difficulty which besets the detection of bacteriocin producers has been the relationship between the phenomena of bacteriocinogeny and lysogeny (Hardy, 1975; Mayr-Harting et al., 1972). Gratia (1932) distinguished between a phage and bacteriocin by titrating the supernatant under test on a lawn of the sensitive strain. In the case of a phage, the dilutions would show a decreasing number of discrete phage plaques; a bacteriocin on the other hand, would show a diffuse thinning of growth which became less marked with increasing dilution of the supernatant (Mayr-Harting et al., 1972).

Methods for screening the bacteriocin producers include the double- or triple-layer method developed by Gratia (1925). Here, the test and indicator culture are grown simultaneously and the demonstration of antagonism is dependent both on the diffusibility of the inhibitor early in the growth of the indicator strain and the density of the indicator lawn. The lawn is usually seeded before inoculation of test strains (Tagg et al., 1973) or may be sprayed onto the surface subsequent to inoculating the potential antagonists (Hsu & Wiseman, 1967; Jetten & Vogels, 1973; Jetten, Vogels & De Windt, 1972). In this laboratory,

lawns were seeded and test strains spotted onto the seeded agar plates.

2.2 METHODS

2.2.1 Media and Chemicals as given in the Appendix.

2.2.2 Anaerobic techniques

The anaerobic glove cabinet (Plate 1) constructed by Moodie & Woods (1973b) was initially used for the anaerobic bacteria described in this chapter. These bacteria were found, however, to be moderately aerotolerant in that they could withstand exposure to air for up to 30 min without loss in viability. As a result, all manipulations could be done on the open bench and care was taken not to expose the bacteria to air for periods longer than 20 min. Large perspex anaerobic jars designed by Moodie & Woods (1973) were used for incubation and storage of cultures (Plate 1). Anaerobiosis was achieved by flushing the jars with CO_2 and H_2 passed through the Deoxy catalyst, for up to 60 min. Palladium catalyst pellets, approximately 5 g per jar, were used to convert any free oxygen to water. Dehydrated silica gel, approximately 500 g per jar, was used to dry freshly prepared plates and a resazurin indicator was used to check that the interior remained anaerobic.

Stock cultures of the bacterial strains were maintained anaerobically on Brain Heart Infusion Agar (BHI-A) slopes and were viable for up to 12 months. In addition, duplicate stock cultures were streaked onto fresh BHI-A plates and kept anaerobically. The cultures on plates were viable for at least 4 weeks and were subcultured every 4 weeks onto fresh BHI-A plates.

2.2.3 Bacterial strains

Obligate anaerobic bacteria were isolated from clinical specimens in the King Edward VIII Hospital in Durban, South Africa. Samples were quickly transferred to anaerobic containers, diluted into Brain Heart Infusion Broth (BHI-B), and spread onto kanamycin and vancomycin blood/agar plates which are selective for Bacteroides species (Finegold, Sugihara & Sutter, 1971). Species identification was carried out at the King Edward VIII Hospital and confirmation to species rank of 5 of the strains was done by Professor L.V. Holdeman, Virginia Polytechnic Institute and State University, Anaerobe Laboratory, Virginia, U.S.A. Details of the strains are shown in Table 2.1.

Isolation and Detection of Bacteriocinogen Strains

A modification of the spot test technique described by Mayr-Harting, Hedges & Berkely (1972) was used. Thirty six Bacteroides strains were tested for their ability to produce or be sensitive to, bacteriocins. The thirty six strains were grown overnight in BHI broth. Samples (0.2 ml) of the overnight cultures of each strain were added to wells in a sterile microtitre plate. This served as a master for the replication of each of the strains onto individual indicator plates. Replication was carried out with a special pronged replicator which was dipped into the wells containing the cultures and then spotted on the surface of seeded double agar layer plates. Bacteriocinogenic strains were detected by the inhibition of growth of the background strain around the test colony (Plate 1). Colonies surrounded by a halo of inhibition of the indicator strain were picked and subcultured.

2.3 RESULTS

2.3.1 Bacterial strains

Of the 36 Bacteroides strains isolated, 27 were classified as Bacteroides fragilis, 2 as Bacteroides melaninogenicus, 3 as Bacteroides ruminicola, 1 as Bacteroides distasonis, 1 as Bacteroides thetaiotaomicron. No firm species identification was obtained for the 2 remaining Bacteroides strains (Table 2.1).

2.3.2 Detection of Bacteriocinogenic strains

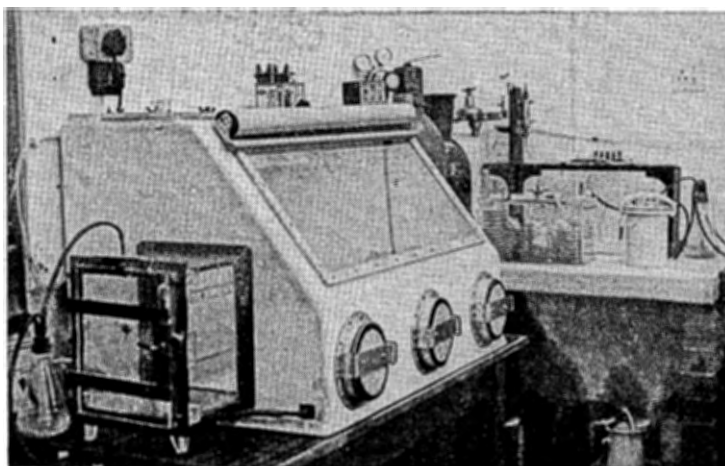
Of the 36 Bacteroides strains, 20 were found to produce bacteriocin-like antagonism which inhibited growth of at least one of the strains tested. The type of zones produced by these colonies could be divided into 2 classes; class I zones were large and clear indicating complete inhibition, whereas class II zones were turbid (Table 2.2).

The strains themselves could be divided into four groups (Table 2.2). Group A strains were very strong producers with the widest spectrum of activity. Strains in this group were mostly resistant to the other bacteriocins produced. Group B strains were weak producers and produced mainly class II zones compared with group A strains which produced mainly class I zones. Another characteristic of group B strains was that they were mostly sensitive to the bacteriocins produced by other strains. Group C strains did not produce bacteriocins but were sensitive to most of the bacteriocins produced. Group D strains did not produce bacteriocins but were not inhibited by the bacteriocins. It is interesting to note that the four strains in this group have been observed to exhibit strong phase variation (Burt et al., 1978) unlike the other strains which showed either weak or no phase variation (Jones - private communication). The significance of the relationship between the phenomena of bacteriocinogeny and phase variation is still unknown at this stage.

Strain	Designation	Species Rank	Producer-indicator properties
O1	Bf-1	<u>B. fragilis</u>	Producer; class I zone; P ⁺⁺ ; S ⁻
C1	Bf-2	"	Group C strain; S ⁺⁺ ; P ⁻
O2	Bf-3	"	Producer; class I zone; P ⁺⁺ ; S ⁻
F1	Bf-4	"	Producer; class I & II zones; S ⁺⁺
F2	Bf-5	"	P ⁺⁺ ; class I & II; S ⁺
F3	Bf-6	"	Group C strain; P ⁻ ; S ⁺⁺
F4	Bf-7	"	Group B; P [±] ; S ⁺⁺
F5	Bf-8	"	P ⁺⁺⁺ ; S ⁻
F6	Bf-9	"	P ⁻ ; S ⁺⁺
F7	Bf-10	"	P ⁻ ; S ⁺⁺
F8	Bf-11	"	P [±] ; S ⁺⁺
F9	Bf-12	"	P [±] ; S ⁺⁺
F10	Bf-13	"	P [±] ; S ⁺⁺
F11	Bf-14	"	P ⁻ ; S ⁻ x Phase variation ⁺
F12	Bf-15	"	P ⁻ ; S ⁻ x Phase variation ⁺
F13	Bf-16	"	P ⁻ ; S ⁺⁺
F14	Bf-17	"	P ⁻ ; S ⁺⁺
F15	Bf-18	"	P ⁻ ; S ⁺⁺
F16	Bf-19	"	P ⁺⁺ ; S ⁻
F17	Bf-20	"	P [±] ; S [±]
F18	Bf-21	"	P [±] ; S [±]
F19	Bf-22	"	P ⁻ ; S ⁻ x Phase variation ⁺
F20	Bf-23	"	P ⁺ ; S ⁻
F21	Bf-24	"	P [±] ; S [±]
F22	Bf-25	"	P ⁻ ; S [±]
F01	Bf-26	"	P ⁻ ; S ⁺⁺
F02	Bf-27	"	P ⁻ ; S ⁺⁺
D1	Bd-1	<u>B. distasonis</u>	P ⁻ ; S [±]
M1	Bm-1	<u>B. melaninogenicus</u>	P ⁺ ; S ⁻
A1	Bm-2	"	P ⁻ ; S ⁺⁺
R1	Br-1	<u>B. ruminicola</u>	P ⁺ ; S ⁺
R2	Br-2	"	P ⁺ ; S ⁺
R3	Br-3	"	P [±] ; S ⁺⁺
T1	Bt-1	<u>B. thetaiotaomicron</u>	P ⁻ ; S ⁻ x Phase variation ⁺
S1	Bx-1	species rank unknown	P ⁻ ; S ⁺⁺
FM1	Bx-1	" " "	P ⁺⁺ ; S ⁻

P⁺ = Producer (bacteriocin⁺) strain P⁻ = non-producer P⁺⁺ = strong producer
 S⁺ = Sensitive (bacteriocin sensitive) S⁻ = non-sensitive strain
 S⁺⁺ = Sensitive to more than 5 bacteriocin⁺ strains

(a) Anaerobic glove box



(b) Anaerobic incubating container

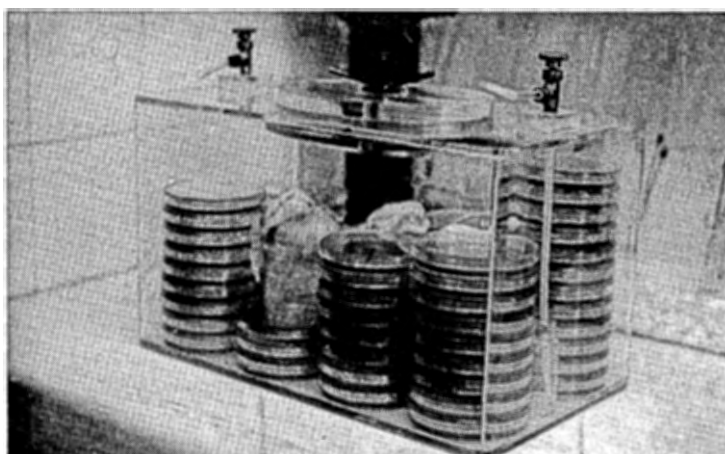
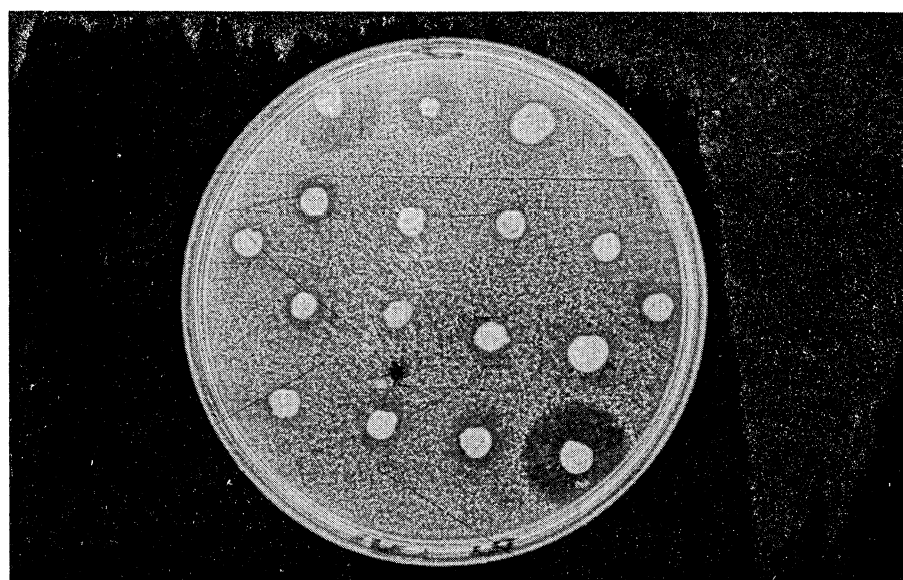


PLATE 2

Bacteriocin zones obtained by detection method described above



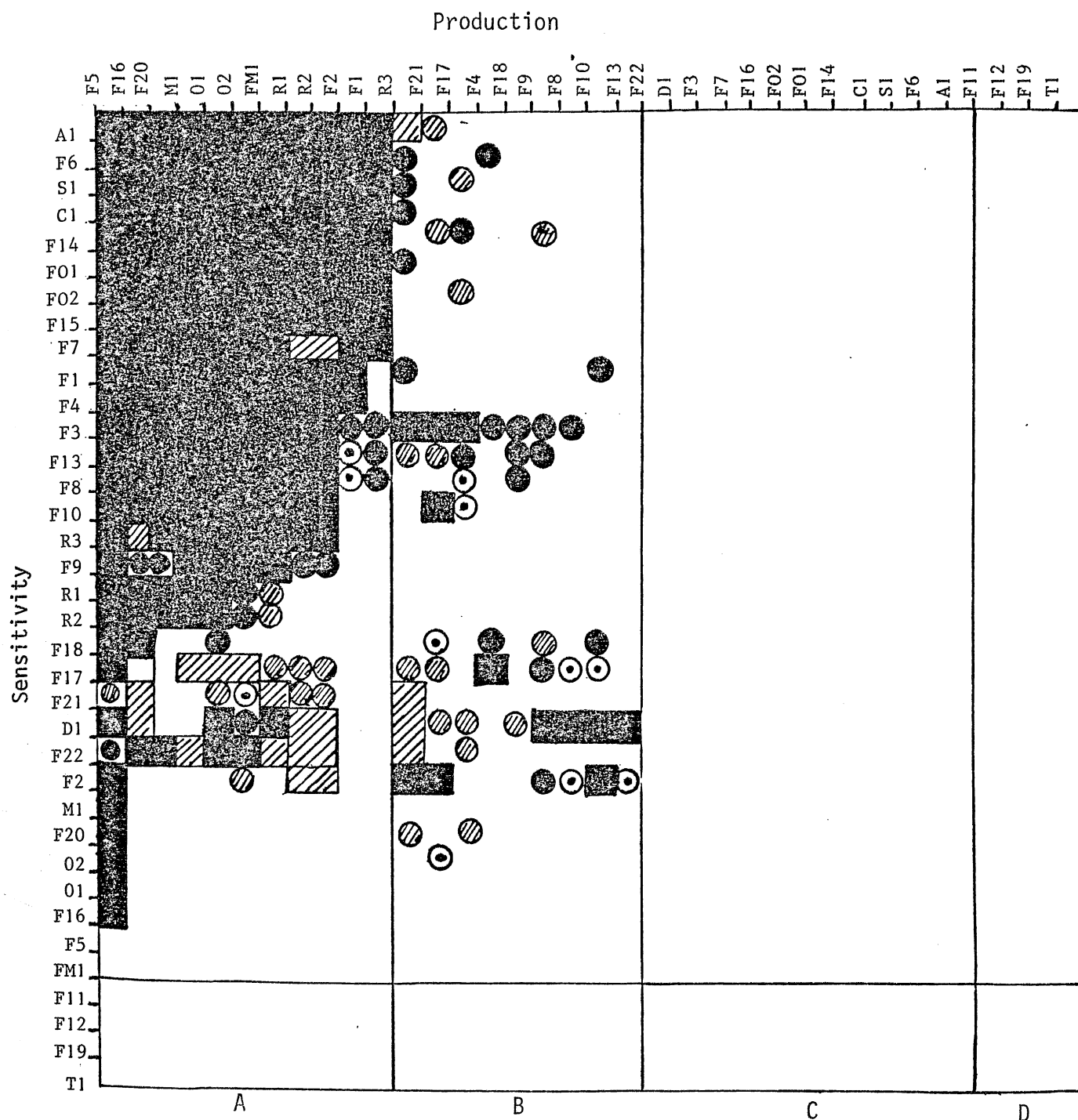


Table 2.2 Bacteriocin production and sensitivity of *Bacteroides* strains. Strains were screened for production of and sensitivity to bacteriocins as described in methods. Strains in the horizontal axis were grouped according to their ability to produce bacteriocins (e.g. F5, F16 ... F1 are strong producers and make group A strains^{*}). The strains in the vertical axis have been arranged according to their sensitivity to produced bacteriocins (e.g. strain C1 is sensitive to bacteriocins produced by strains F5, F16 ... F1 etc).

^{*}(Strain F5 produces a bacteriocin which inhibits growth of strains A1, F6, S1, C1 ... F16 on vertical axis).

2.3.3 Choice of producer and sensitive strains

Two Bacteroides fragilis strains, one a producer strain and the other a sensitive indicator strain were selected for further study. These strains were designated Bf-1 and Bf-2 for the producer and sensitive strains, respectively, and this designation is used throughout this thesis.

2.3.4 Growth kinetics of Bf-1 and Bf-2

The growth characteristics of B. fragilis strains Bf-1 and Bf-2 were determined and found to be similar under identical conditions. No drop in turbidity was observed with Bf-1 indicating that production of the bacteriocin was not accompanied by cell lysis

CHAPTER III

PRODUCTION OF Bf-1 BACTERIOCIN

Bacteroides fragilis (Bf-1) strain produces a bacteriocin which is not inducible by either UV irradiation or treatment with mitomycin C. In fact, mitomycin C concentrations above $0.4 \mu\text{g ml}^{-1}$ inhibited bacteriocin production. This was probably due to the poor growth of the producer cultures. The bacteriocin is produced towards the end of the exponential phase of growth and maximum bacteriocin titres are obtained at the beginning of stationary phase. Prolonged incubation decreased the bacteriocin titres to 25% of maximum, after 24 h.

3.1 INTRODUCTION

Reeves (1965) wrote

Bacteriocinogenic strains although they possess the stable genetic ability to produce a bacteriocin, do not do so all the time and under all conditions. The factors controlling the synthesis are only incompletely understood.

This still holds true and only a few workers have systematically studied the cultural conditions for bacteriocin production (Mayr-Harting, Hedges & Berkely, 1972).

The conditions for production of bacteriocins appear to vary from one strain to another. In a number of instances significant production of bacteriocins has been demonstrated only on solid or semi-solid media (Nicolle & Prunet, 1964; Holland & Roberts, 1964; Gangliano & Hinsdill, 1970). Although synthesized in liquid culture, production of

staphylococcin 1580 can be increased by growth in semi-solid medium (Jetken, Vogels & De Windt, 1972). Particular medium components may be found to be critical for the production of individual bacteriocins. Butyricin 7423 production was enhanced by the addition of certain amino acids (Clarke et al., 1976) and manganese ions were found to be necessary for megacin production (Alföldi, 1958). It seems likely, therefore, that optimum conditions for the production of bacteriocins are those which allow optimum expression of the bacteriocinogenic factors. In fact, Herschman and Helinski (1967) reported that derivatives of Escherichia coli W3110, one colicinogenic for E2 and the other for E3, gave highest colicin yields under very different conditions.

The time of incubation is also important for the maximum bacteriocin titres to be obtained for some bacteriocins (Halbert & Magnuson, 1948; Dajani, Tom & Law, 1976; Mayr-Harting et al., 1972) but not for others (Papavassiliou, 1963; Farkers-Himsely & Seyfried, 1963). Streptocin STH, production was shown to be best during the exponential growth phase, with a sharp decline in the level of the bacteriocin just before the culture entered stationary phase (Schlegel & Slade, 1973). By contrast, streptococcin A-FF22 production starts late in the exponential phase and activity slowly decreases on prolonged incubation (Tagg et al., 1976). Colicins E2 and E3 production was not affected by the period of incubation (Reeves, 1972). It has also been shown that conditions such as variation in temperature (Dajani & Taube, 1974; Tagg, Dajani & Wannamaker, 1975), aeration (Jetten Vogels & De Windt, 1972), and pH (Goebel, Barry, Jesaitis & Miller, 1955) have profound effects on the yield of the active bacteriocin.

Since the discovery by Jacob et al. 1952) that colicin synthesis can be induced by ultraviolet light, other agents which affect DNA replication were found to induce bacteriocin production. Mitomycin C - an antibiotic isolated from Streptococcus caespitosus, is the most commonly used inducing

inducing agent. Thymine starvation (Foulds & Shemin, 1969), transient inhibition of protein synthesis (Ben-Gurion, 1970) and various mutagenic agents (Lwoff, Jacob, Ritz & Gage, 1952) are also used. Gromkova (1971) reported the induction of colicin Ia synthesis at elevated temperatures. Cold-shock treatments have been shown to induce greater yields of Vibrio comma bacteriocin from organisms grown under thioglycollate-produced anaerobic conditions (Farkas-Himsley & Segfried, 1963).

The bacteriocin from Bacteroides fragilis (Bf-1) strain is produced in an extracellular form in liquid media. Production starts towards the end of the exponential growth phase and is maximum for 2 hours after the beginning of stationary phase. In an attempt to increase the yield, different growth and inducing conditions were studied in order to obtain sufficient amounts for purification studies.

3.2 METHODS

3.2.1 Media and chemicals as given in Appendix.

3.2.2 Preparation of sample for assay

Bacteriocin producer cells were centrifuged at 10 000 g for 10 min at 4°C. The supernatant was then stored at 4°C over 10% chloroform to kill any residual bacteria. The crude supernatant was found to be relatively stable when stored at 4°C over chloroform.

3.3.3 Bacteriocin assay

Samples (0.1 ml) of an overnight BHI-broth indicator culture were added to molten 10 ml BHI-agar (1% agar w/v) at 50°C and the contents thoroughly mixed before being poured into a sterile petri dish. After

the agar had solidified, the plates were gassed for 20 min in an anaerobic jar using the $\text{CO}_2 + \text{H}_2$ mixture. Wells were then punched into the agar using a sterile cork-borer (5 mm diameter). A series of two-fold dilutions of the test culture supernatants were made and 25 μl of each dilution was added to each well. The plates were then re-gassed and incubated at 37°C for 24 h. The bacteriocin activity in arbitrary units (AU) was defined as the reciprocal of the highest dilution which gave a completely clear zone of inhibition.

3.2.4 Production kinetics

An overnight BHI-broth producer culture was diluted 1:10 in BHI-broth, and incubated at 37°C . At different time intervals, aliquots of the culture were harvested, centrifuged at 10 000 g for 10 min at 4°C , sterilized with chloroform and assayed for bacteriocin.

3.2.5 Location of bacteriocin

The pellets (cells) obtained as described above were treated as follows: 1 M sodium chloride (NaCl) was used to wash the cells and the supernatant assayed for bacteriocin activity. The NaCl treated cells were then resuspended in minimum amount of 0.02 M Tris-HCl buffer, pH 8.2 and disintegrated by sonication at an amplitude of 8 microns using an MSE sonicator for 2 min. The temperature was not allowed to rise above 4°C during this process. The disintegrated cells were then centrifuged at 24 000 g for 15 min. The supernatant was sterilized with chloroform and assayed for bacteriocin.

3.2.6 Induction of bacteriocin

Mitomycin C (0.1; 0.2; 0.3; 0.4; 0.6; 0.8 and $1.0 \mu\text{g ml}^{-1}$ concentration) was added to 10 ml samples of the producer culture of an

absorbance $A_{600 \text{ nm}}$ ca. 0.20. The cultures were incubated anaerobically and assayed for bacteriocin after 6 and 24 h. Induction by ultraviolet light was determined by exposing 7.5 ml samples of exponentially growing producer cells to a germicidal lamp ($0.45 \text{ Joules m}^{-2} \text{ sec}^{-1}$) for 0, 20, 40 and 60 sec. The irradiated cultures were centrifuged and the pellets resuspended in fresh BHI-broth and incubated anaerobically before assaying for bacteriocin after 6 and 24 h.

3.3 RESULTS

3.3.1 Effect of growth media on bacteriocin synthesis

The effect of media on bacteriocin production was determined in BHI-broth, peptone-yeast extract and glucose minimal media. Bacteriocin titres could only be detected in complex media, highest on BHI-broth media.

3.3.2 Production kinetics in liquid media

The bacteriocin was first detected in the supernatants of cultures in mid-exponential phase and the maximum bacteriocin titres were extracellular and were obtained at the beginning of the stationary growth phase (Fig 3.1). This titre decreased by 50% 2 h after the maximum titres were obtained.

3.3.3 Location of bacteriocin

Most of the 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. Similarly, disrupting the cells by sonication did not result in any significant increase in bacteriocin titres (Table 3.1).

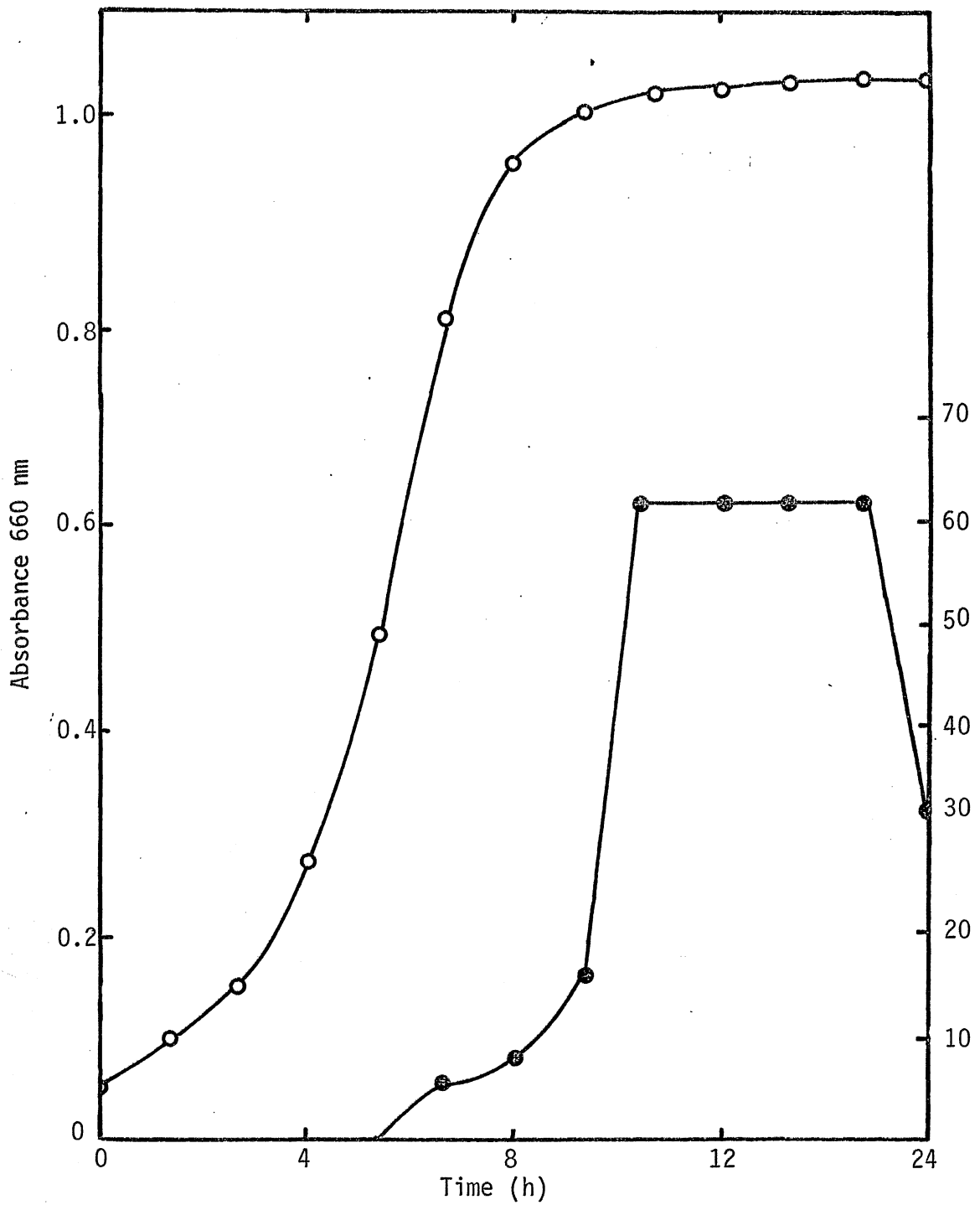


Fig. 3.1 Production of bacteriocin in BHI-broth. Absorbance 660 nm (o); bacteriocin activity in AU (●).

Table 3.1 Localization of the bacteriocin

Time of production (h)	Turbidity	Bacteriocin activity (AU)		
		Extracellular ^a	Cell bound ^b	Intracellular ^c
5	0.74	8	2	1
6	0.85	16	2	1
7	0.95	32	2	1
8	1.00	64	2	1
10	1.06	64	2	1
12	1.08	32	2	1
24	1.10	16	2	2

^a Culture supernatant

^b After centrifugation of the culture at 10 000 g for 10 min at 4°C, pellets were washed with 1 M NaCl, re-centrifuged and the supernatant assayed

^c Sonicated pellets

3.3.4 Induction of Bacteriocins

Neither mitomycin C nor exposure to ultraviolet light induced bacteriocin synthesis. (Tables 3.2(a) and 3.2(b)). Higher mitomycin C concentrations were shown to decrease the final bacteriocin titres probably due to inhibition of cell growth.

3.4 DISCUSSION

The bacteriocin produced by Bacteroides fragilis was unstable in the liquid culture in which it was produced. Maximum titres were obtained for only 2 h during the incubation period and there was a gradual decrease in titre with time. This could be due to the release in the medium of a bacteriocin inhibitor or inactivator. Davie & Brock (1966) found that a specific teichoic acid, usually contained within the cell envelope of S. faecalis subsp symogenes strain X14, contributed to the immunity of this organism to its own bacteriocin (hemolysin). Only strains resistant to this lytic agent produced the specific inhibitor. Excretion of some of the inhibitor in the later stages of the growth cycle resulted in the apparent disappearance of the bacteriocin from the cultures. Moreover, all of the bacteriocins characterized to date contain a protein component that is essential for biological activity. Irreversible inactivation of some bacteriocins may thus result from digestion by proteases of which are also produced by the organism as is the case with Serratia marcescens (Foulds & Shemin, 1969) and Clostridium botulinum (Ellison & Kauffer, 1971).

The bacteriocin is not inducible by both UV light and mitomycin C. This is not unusual since Clarke, Robson & Morris (1975) and Tubylewicz (1976) reported the non-inducible nature of bacteriocins produced by Clostridium

Table 3.2(a) Induction using mitomycin C

	Mitomycin C ($\mu\text{g ml}^{-1}$)						
	.0	0.1	0.2	0.4	0.6	0.8	1.0
Activity (AU)	46	64	64	32	32	16	8

Mitomycin C was added to cultures $A_{660\text{nm}}$ ca. 0.2 and incubated for a further 6 h.

Table 3.2(b) UV Induction

Irradiation time (sec)	Activity (AU)	
	a	b
0	60	30
20	60	30
40	60	30
60	60	30

^a Cultures assayed after incubation for 6 h after irradiation

^b Cultures incubated for 22 h after irradiation

species. There are other examples where the bacteriocin yield appears to be reduced on induction (Dajani, Gray & Wannamaker, 1970; Hongo, Murata, Kono & Kato, 1968; and Mahony & Butler, 1971). Since most of these strains are anaerobes to a lesser or greater extent, there may be a correlation between their anaerobic nature and the ability of their bacteriocinogenic factors to be induced by the known inducing agents. Since the bacteriocin is produced in an extracellular form there could be a closer relationship between the bacteriocinogenic plasmid of B. fragilis with Mor 17A (a Proteus morganii bacteriocinogenic plasmid), Col E⁺ and Col K⁺ than with Col B, Col I or Col II plasmids whose bacteriocins are tightly bound to the cell membrane (Williams, 1977; Hardy, 1975).

CHAPTER IV

CHARACTERISTICS OF THE Bf-1 BACTERIOCIN

The extracellular bacteriocin produced by Bacteroides fragilis is stable between pH 7.5 and 8.2. The majority of the bacteriocin is thermolabile but a small proportion (ca. 3%) is stable after autoclaving at 121°C for 15 min. The activity of this compound is not affected by deoxyribonuclease (DNAase) or ribonuclease (RNAase) but is rapidly lost on incubation with either trypsin or pronase. Partial purification by precipitation with $(\text{NH}_4)_2 \text{SO}_4$, followed by chromatography through DEAE-cellulose and Sephadex G-100 gave a single peak and a molecular weight estimate of 13 500 daltons was obtained from Sephadex G-100 chromatography. SDS-PAGE gave a MW estimate of 18 790.

4.1 INTRODUCTION

A lot of data has been obtained about bacteriocins by studying the physico-chemical properties of the crude (unpurified) preparations. Evidence to the gross chemical nature of bacteriocins is easily obtained by using enzymes. It was on this basis that streptocin STH₂ was thought to be a complex molecule containing essential protein, lipid and phosphate groups (Schlegel & Slade, 1973). Colicins have been shown to be simple proteins with molecular weights of between 18 000 and 90 000 (Hardy, 1975). Most bacteriocins have also been shown to be simple proteins (Holland, 1961; Hardy, 1975).but a few others, including certain staphylococcal (Gangliano & Hinsdill, 1970; Hale & Hinsdill, 1973) and lactobacillus (de Klerk & Smit, 1967; Upreti & Hinsdill, 1973) seem, in their present state of purification, to be quite complex molecules with lipid and carbohydrate components, in addition to protein. The purification of a bacteriocin

depends on its location at the time of harvest. Since it is not usually possible to obtain a sufficiently concentrated source of bacteriocin for purification, it is usually necessary to start with large batches of culture. Using large culture volumes has its disadvantages, especially if the bacteriocin is produced in an extracellular form. In this case one has to be content with purifying the bacteriocin from the contaminating supernatant compounds. Where the bacteriocin is bound to the cell wall there is an advantage in that supernatant fluid contaminants are eliminated although some activity might be lost through extraction of pelleted cells. The extraction procedures utilized include washing with 7 M urea (Hale & Hinsdill, 1973) for staphylococcin P62 and with 5% NaCl for staphylococcin 1580 (Jetten, Vogels & De Windt, 1972). Colicins E2-W3110 and E3-W3110 (Herschman & Helinski, 1967) and a bacteriocin from Serratia marcescens (Foulds, 1972) were extracted by similar procedures. With bacteriocins which are located intracellularly or are cell-bound but cannot be extracted by changing the ionic conditions of the medium, a different approach is used. Cells are pelleted, washed to remove extracellular contaminants before being disrupted either chemically or mechanically. Staphylococcin 414 could be released from the cell wall by mechanical disruption of the cells (Gangliano & Hinsdill, 1970) and this method has also been applied to the purification of colicins Ia and Ib (Konisky & Richards, 1970), colicin D (Timmis, 1972), colicin B (Braun, Schaller & Wabl, 1974) and cloacin DF 13 (De Graaf, Goedvolk-de-Groot & Stouthamer, 1970).

Since most bacteriocins are of a protein nature, most purification methods used with enzymes and other proteins have been employed with great success with various bacteriocins. De Klerk & Smit (1967) used a rotary evaporator at 40°C to concentrate a Lactobacillus fermenti bacteriocin before purifying by chromatography on Sephadex G-100. Goebel & Barry (1958)

concentrated colicin K by distillation in vacuo at below 20°C. Precipitation by $(\text{NH}_4)_2 \text{SO}_4$ (Reeves, 1963; Sandoval et al., 1965) and ethanol (Hinsdill & Goebel, 1964) has been used to concentrate some bacteriocins. Other purification methods used include chromatography through DEAE-cellulose (Kageyama & Egami, 1962; Rude & Goebel, 1962; Reeves, 1963) and molecular exclusion chromatography using Sephadex G-100 or G-200 (Dandeu & Barbu, 1967; De Klerk & Smit, 1967). Iso-electrofocusing has also been used to purify and check the purity of some bacteriocins (Timmis, 1972; Williams, 1976). The Bf-1 bacteriocin was characterized and purified using the established techniques described.

4.2 METHODS

4.2.1 Media and Chemicals are listed in the Appendix.

4.2.2 Preparation of Bf-1 bacteriocin

A sample (20 ml) of an overnight BHI-broth producer culture was used to inoculate 2 litres of anaerobic BHI-broth in an anaerobic system. The flask was then gassed for a further 30 min before being incubated at 37°C for 9 h. The cultures were centrifuged at 10 000 g for 10 min at 4°C, and the supernatant stored over 10% chloroform at 4°C to kill any residual bacteria.

4.2.3 Temperature stability

Samples (2 ml) of the crude supernatant were incubated at the following temperatures for 60 min: 4, 18, 37, 50, 60, 80 and 100°C. At 5 min intervals, 25 μl aliquots were withdrawn from each sample at the different temperatures and assayed for bacteriocin activity. Furthermore, 2 ml of the

crude supernatant was autoclaved at 121°C for 15 min and then assayed for bacteriocin activity. The stability of the bacteriocin in the crude supernatant was determined by assaying samples held at 4°C every 24 h for 7 days.

4.2.4 pH Stability

Using appropriate buffers, the stability of the bacteriocin at pH values between 2.7 and 10.5 was determined by mixing equal volumes of the crude supernatant and buffer and incubating at 37°C for 2 h before assaying for activity.

4.2.5 Sedimentation of the bacteriocin

The bacteriocin sample was centrifuged at $150\,000\text{ g}$ for 2 h. The supernatant fluid and the pellet which was dissolved in a minimum amount of buffer were assayed for bacteriocin activity.

4.2.6 Chemical properties of the bacteriocin

DNAase and RNAase ($50\text{ }\mu\text{g ml}^{-1}$) were each dissolved separately in 0.02 M Tris-HCl, pH 8.2. Equal volumes of each enzyme and bacteriocin were mixed and incubated at 37°C for 2 h. The effect of chloroform (50% v/v) was also determined by the method described above. Samples were then assayed for bacteriocin activity.

4.2.7 Partial purification of the bacteriocin

The bacteriocin was purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$, followed by chromatography through DEAE-cellulose and Sephadex G-100. All purification steps described below were performed at 4°C . Two litres of crude supernatant were prepared as described above. Protein concentration was determined by the method of Lowry *et al.* (1951).

4.2.7.1 Ammonium sulfate precipitation

The crude lysate was brought to 30% saturation by slow addition, with stirring, of solid $(\text{NH}_4)_2 \text{SO}_4$. Stirring was continued for 4 h, after the last addition of salt, and the precipitate was removed by centrifugation at 12 000 g for 20 min at 4°C, and discarded. The supernatant fluid was then brought to 60% ammonium sulfate saturation and stirring was continued overnight at 4°C after the last addition of salt. The 30 - 60% $(\text{NH}_4)_2 \text{SO}_4$ precipitate fraction was collected by centrifugation, dissolved in 0.02 M tris-HCl buffer, pH 8.2 and dialysed against three changes of 0.02 M tris-HCl, pH 8.2 and assayed for bacteriocin activity. Protein concentration was determined by the Lowry method (1951).

4.2.7.2 DEAE-cellulose chromatography

The dialysed 30 - 60% $(\text{NH}_4)_2 \text{SO}_4$ fraction was then loaded onto a column of DEAE-cellulose (2.5 x 36 cm) equilibrated with the tris buffer. The column was then washed with 200 ml of tris-HCl buffer, and the protein eluted with a 500 ml (2 x 250 ml) linear 0 - 0.30 KCl gradient. Both components of the gradient contained the tris-HCl buffer. Fractions containing the highest specific activity were pooled, assayed for bacteriocin and protein, and concentrated by freeze-drying.

4.2.7.3 Sephadex G-100 chromatography

The freeze-dried DEAE-cellulose pool was dialysed against the tris-HCl buffer overnight at 4°C. This fraction was then loaded onto a Sephadex G-100 column (1 x 30 cm) equilibrated with the tris-HCl buffer. The void volume was determined with 0.3 ml blue dextran

2000 (2×10^6 daltons) at a flow rate of 8 ml/h. The column was calibrated with bovine serum albumin (MW 67 000 daltons), ovalbumin (MW 43 000 daltons), cytochrome C (12 400) and insulin (6 000).

4.2.7.4 SDS-Polyacrylamide gel electrophoresis

The method of Shapiro et al. (1967) for molecular weight estimation and purity determination using polyacrylamide gels was employed. The following stock solutions were prepared:

- A. 6% cyanogum 41 in 0.1 M sodium phosphate buffer, pH 7.2
- A¹. 6% cyanogum 41 in 0.1 M borate buffer, pH 8.6
- B. 10% SDS
- C. 10% 2-mercaptoethanol
- D. 0.3% dimethylamino-propionitrile (DMAP)
- E. 5% ammonium persulfate
- F. 0.25% Coomassie blue in methanol : water : acetic acid (9 : 9 : 2)
- G. 0.10 M sodium phosphate buffer pH 7.2 containing 0.1% SDS and 0.1% 2-mercaptoethanol

Solution A (20 ml) was degassed by evacuation for at least 1 h with vigorous shaking before being allowed to equilibrate at 40°C for 10 min. Solution B (0.2 ml) and solution C (0.2 ml) were then added with gentle mixing.

Polymerization was initiated by the addition of 0.1 ml solution D and 0.25 ml solution E. The mixture was then used to fill vertical tubes, with one end sealed with Parafilm, to about 1.2 cm from the top, before being overlaid with 0.05 ml 0.1 M phosphate buffer, pH 7.2.

The tubes were then placed in the rubber grommets of the electrophoresis tank and the 2 electrode vessels filled with solution G. Using a Hamilton syringe, protein markers and bacteriocin samples (10 μ l) prepared by boiling for 3 min in the presence of 0.1% SDS, 0.1% 2-mercaptoethanol and 10% glycerol were layered onto the surfaces of the gels. The initial current of 4 mAmp per tube was increased to 10 mA per tube when the samples had entered the gel. The samples were electrophoresed for 2 h. Gels were freed from the glass by gentle rimming with a needle and syringe filled with distilled water. Solution F was poured over the gels in petri dishes and staining allowed to continue for 4 hours before destaining in 10% acetic acid. The following protein markers were used: lysozyme (10 mg ml⁻¹), bovine serum albumin (10 mg ml⁻¹) and β -lactoglobulin (10 mg ml⁻¹) were prepared in phosphate buffer.

4.3 RESULTS

4.3.1 Temperature stability

The bacteriocin was stable for 60 min at 37°C but partially and rapidly inactivated at 60°C ($t_{1/2}$ = 2.5 min at 60°C). However, the bacteriocin was not completely inactivated by heating at 60°C for 60 min and retained 3% of its activity. The 3% fraction was not inactivated by autoclaving at 121°C for 15 min. The pattern of thermal inactivation of the bacteriocin at different temperatures is shown in Fig 4.1.

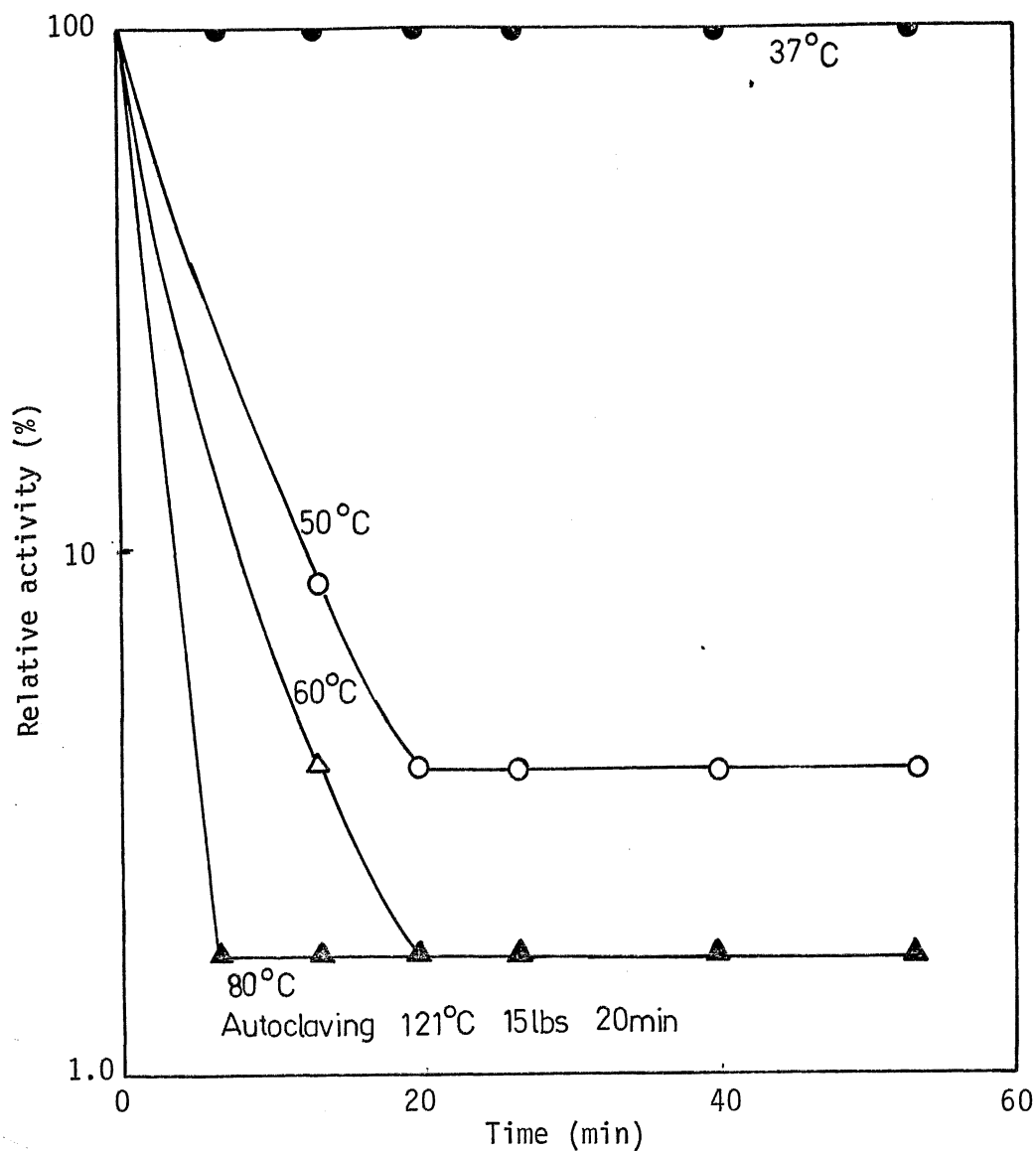


Fig. 4.1 Thermal inactivation of the Bf-1 bacteriocin. Samples held at different temperatures for 2 h were assayed for remaining activity.

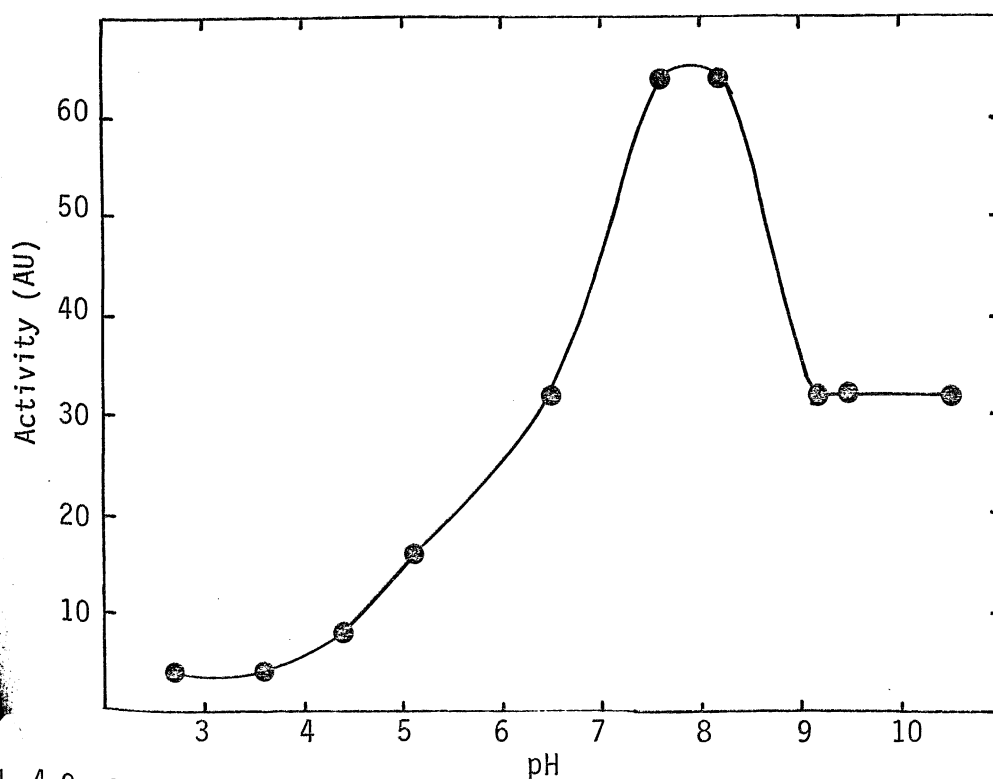


Fig. 4.2 Samples held at 37°C in appropriate buffers for 2 h and assayed for bacteriocin activity.

4.3.2 pH Stability

The bacteriocin was rapidly inactivated on incubation for 2 h at pH values below 5.0. Maximum activity was obtained at pH values between 7 and 9, indicating stability within these pH values. There was a 50% loss of activity at pH values higher than 9.0 (Fig 4.2).

4.3.3 Sedimentation

The bacteriocin was not sedimented by centrifugation at 50 000 g for 2 hours, indicating that it is not a large molecular weight substance.

4.3.4 Chemical properties

Ribonuclease (RNAase) and deoxyribonuclease (DNAase) did not inactivate the bacteriocin. However, both trypsin and pronase inactivated the bacteriocin on incubation for 2 h at 37°C (Table 4.1).

Table 4.1 Inactivation of bacteriocin Bf-1

Treatment	Bacteriocin titre after treatment (AU)	Inactivation (%)
Control	64	-
DNAase (50 $\mu\text{g ml}^{-1}$)	64	0
RNAase (50 $\mu\text{g ml}^{-1}$)	64	0
Pronase (100 $\mu\text{g ml}^{-1}$)	2	96.9
Trypsin (100 $\mu\text{g ml}^{-1}$)	2	96.9
Chloroform (50% v/v)	32	50

4.3.5 Precipitation of the bacteriocin

Acetone could not be utilized for the precipitation of the bacteriocin and no activity was obtained in either the pellets or supernatant after acetone treatment. However, considerable recovery was obtained on precipitation with $(\text{NH}_4)_2 \text{SO}_4$. Fractional precipitation with $(\text{NH}_4)_2 \text{SO}_4$ indicated that most of the bacteriocin (ca. 80% recovery) was obtained after 60% saturation with $(\text{NH}_4)_2 \text{SO}_4$ (Table 4.2).

4.3.6 DEAE-cellulose chromatography

The bacteriocin did not bind very tightly to DEAE-cellulose and only needed a 0 - 0.30 KCl gradient (linear) to elute the compound (Fig 4.3). Thorough washing of the column with the tris-HCl buffer did not elute the bacteriocin which suggested that it had a negative charge. Considerable purification (ca. 46x) was obtained by the $(\text{NH}_4)_2 \text{SO}_4$ and DEAE purification steps (Table 4.3 and Fig 4.3).

4.3.7 Sephadex G-100 chromatography

Elution through a Sephadex G-100 column resulted in a 320 fold purification of the bacteriocin (Table 4.3). Using markers described in the methods, an approximate molecular weight of 13 500 daltons was obtained for the bacteriocin (Fig 4.5). The bacteriocin was eluted as a single peak on Sephadex G-100 (Fig 4.4).

4.3.8 SDS-PAGE

The bacteriocin fraction after Sephadex G-100 chromatography showed a single band on electrophoresis with SDS-polyacrylamide. When the distance of migration between the bacteriocin sample and the 3 protein markers was compared, a molecular weight estimate of 18 790 was obtained (Fig 4.6).

Table 4.2 Fractional $(\text{NH}_4)_2 \text{SO}_4$ precipitation

Treatment	Bacteriocin Titre			
	Vol (ml)	AU/ml	Total AU	% Recovery
Supernatant fluid	100	2 560	2.56×10^5	100
30% $(\text{NH}_4)_2 \text{SO}_4$	10	320	0.03×10^5	0.9
40% $(\text{NH}_4)_2 \text{SO}_4$	10	10 240	1.02×10^5	40
50% $(\text{NH}_4)_2 \text{SO}_4$	10	20 480	2.048×10^5	80
60% $(\text{NH}_4)_2 \text{SO}_4$	10	20 480	2.048×10^5	80

Table 4.3 Purification and Specific Activities of Bacteriocin

Purification step	Recovery (%)	Specific activity (AU/mg)	Purification (x)
Culture supernatant	100	213	-
$(\text{NH}_4)_2 \text{SO}_4$ precipitate	58	1 365	6.4
DEAE, peak	34	9 752	45.7
Sephadex G-100, peak	13	68 270	320.0

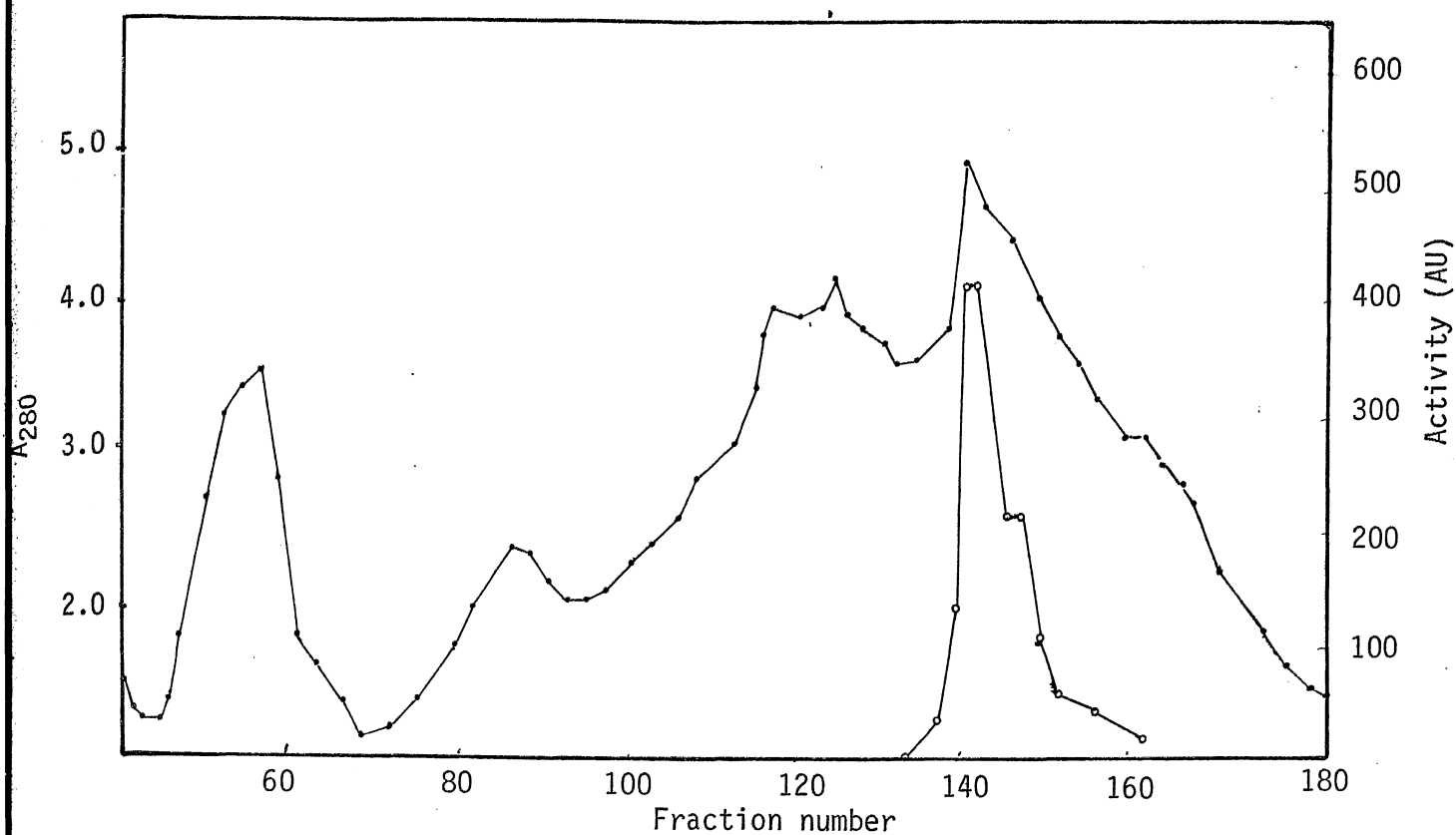


Fig. 4.3 DEAE-cellulose chromatography, pH 8.2 of Bf-1 bacteriocin. A 60 ml sample containing 512 AU of Bf-1 bacteriocin was applied onto the column (●) A_{280} ; (○) bacteriocin activity.

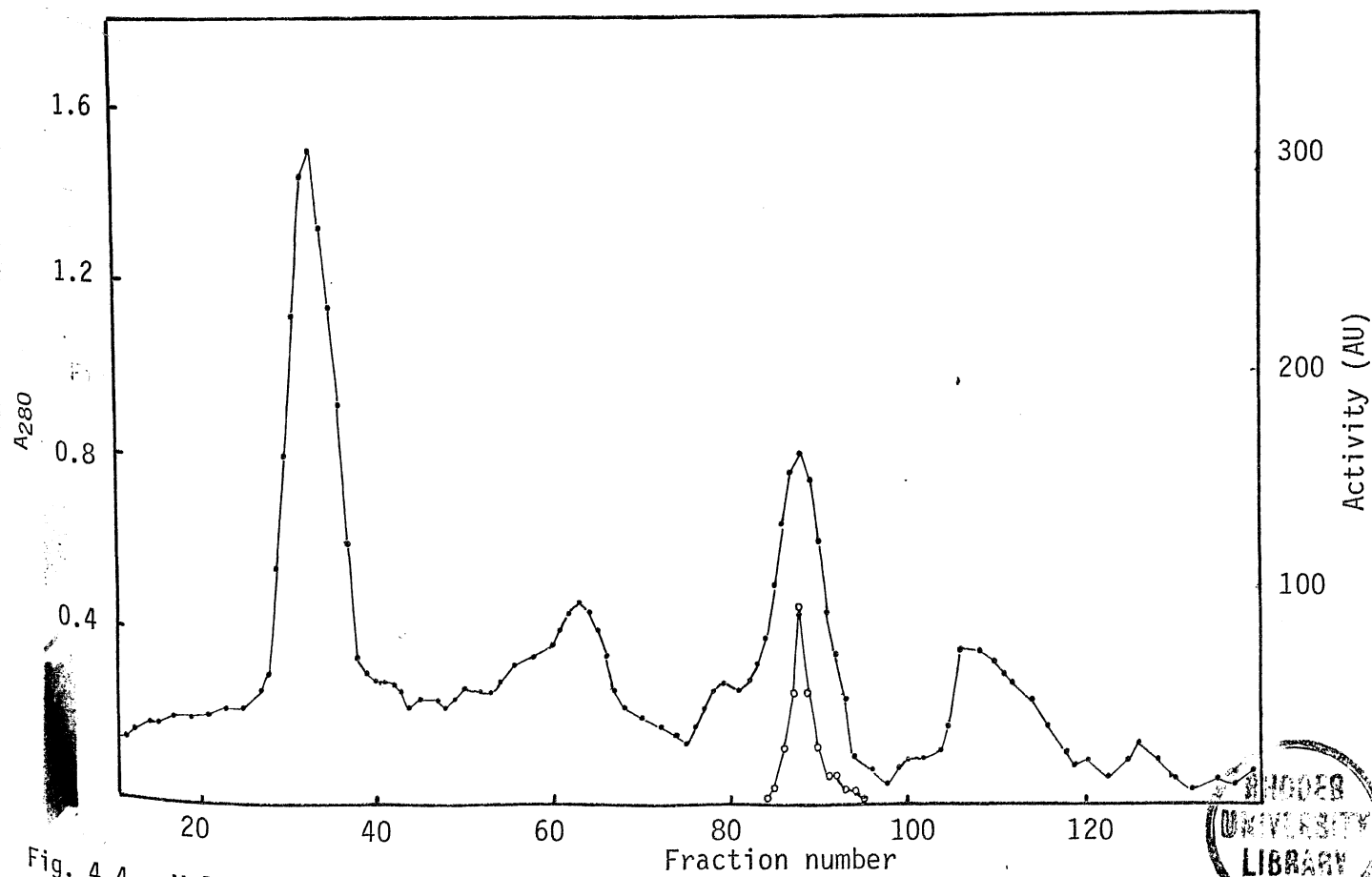


Fig. 4.4 Molecular exclusion chromatography, pH 8.2.



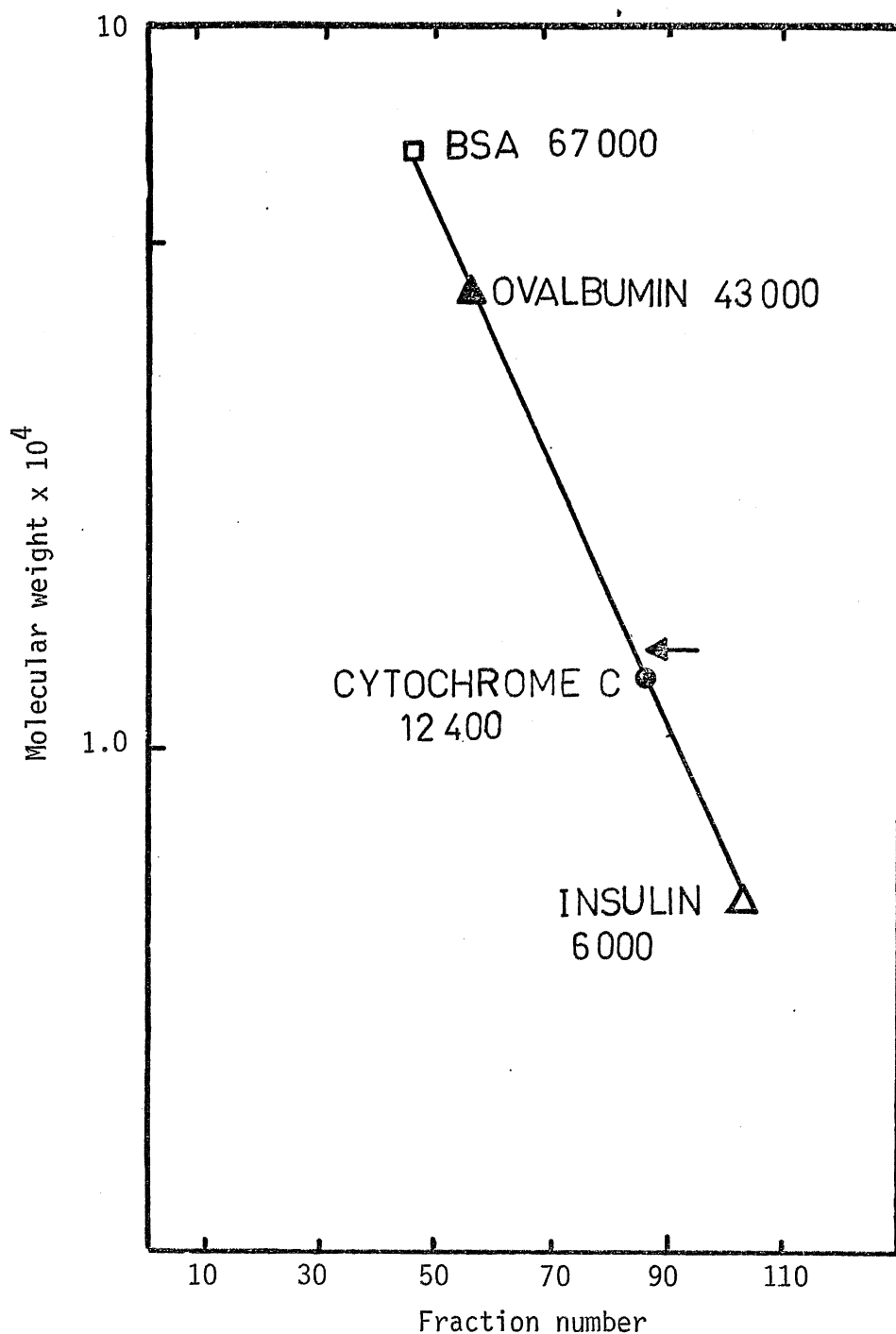


Fig. 4.5 Estimation of the molecular weight of the Bf-1 bacteriocin by gel-filtration on Sephadex G-100. The column was calibrated with bovine serum albumin (BSA - 67 000), ovalbumin (43 000); cytochrome C (12 400); and insulin (6 000). Arrow indicates the position of the bacteriocin.

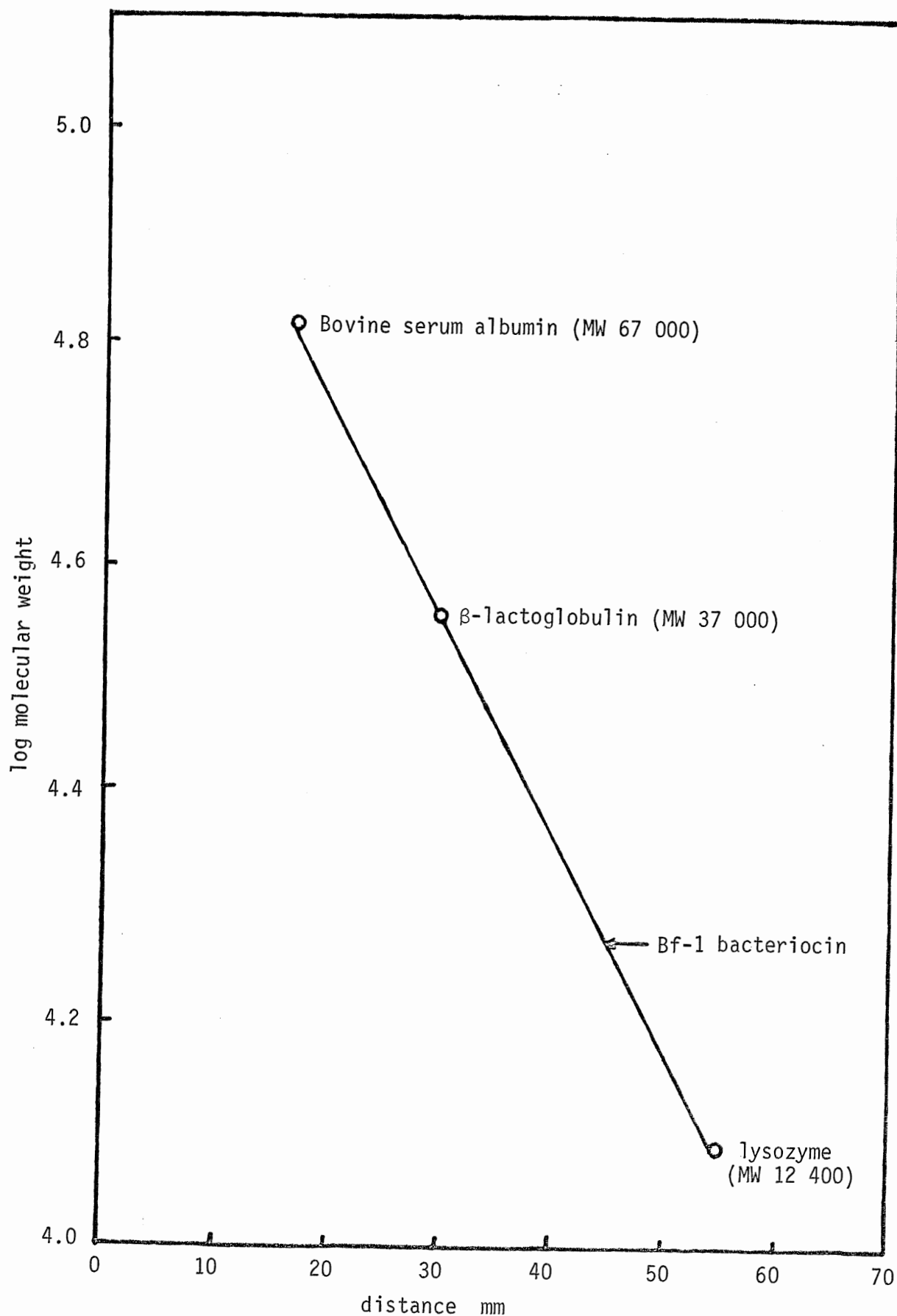


Fig 4.6 Estimation of MW using SDS-PAGE. The protein markers above were treated and run with the bacteriocin sample as described in methods. The arrow indicates the position of the Bf-1 bacteriocin.

4.4 DISCUSSION

Although bacteriocins from Bacteroides species have been reported previously (Beerens & Baron, 1965; Beerens, Baron & Tahon, 1966; Booth, Johnson & Wilkins, 1977; Podhaisky & Reinhold, 1970), not much is known about them. They are similar in that they are all susceptible to proteolysis (Beerens et al., 1966; Podhaisky & Reinhold, 1970; Booth et al., 1977) but differ in their thermostability. Some of the bacteriocins reported are similar to the Bf-1 bacteriocin in that they are heat labile (Beerens & Baron, 1965; Beerens et al., 1966) but one bacteriocin, produced by a Bacteroides strain T1-1 (Booth et al., 1977) is heat resistant and can withstand treatments at 121°C for 1 h without any loss in activity.

The T1-1 bacteriocin described by Booth et al. (1977) is unusual in its temperature stability since the molecular weight estimate reported is in excess of 300 000 daltons (Booth et al., 1977). No less unusual is the temperature stability of the Bf-1 bacteriocin which elutes as a single peak on Sephadex G-100 but has a thermosensitive and thermolabile component. The molecular weight estimate obtained was between 13 500-18 790. These two bacteriocins, T1-1 and Bf-1 do not comply with Bradley's classification of bacteriocins. Bradley (1967) classified the bacteriocins according to their molecular weight and thermostability. Low molecular weight forms are more susceptible to trypsin digestion but are less sensitive to heat inactivation. High molecular weight forms, are on the other hand, resistant to trypsin digestion but more susceptible to heat inactivation.

The biphasic temperature inactivation of the Bf-1 bacteriocin is an unusual feature. While being inactivated by incubation at 60°C for 20 min, a small proportion of its activity is retained even after autoclaving at 121°C for 15 min. While biphasic pH stabilities have been

described for other bacteriocins (Schlegel & Slade, 1973; Williams, 1976), no biphasic temperature stability for bacteriocins has, to my knowledge, been reported. Another unusual feature of the Bf-1 bacteriocin was its sensitivity to acetone and it was in this regard different to the Clostridium acetobutylicum bacteriocin which could be precipitated by both $(\text{NH}_4)_2 \text{SO}_4$ and acetone (Barber et al., 1979).

CHAPTER V

MODE OF ACTION OF Bf-1 BACTERIOICIN

Killing of the sensitive bacteroides cells by Bf-1 follows single-hit kinetics and the interaction of a single molecule of bacteriocin with a target cell occurs in two stages. The killing of sensitive cells is affected by temperature and the growth state of the sensitive cells. The bacteriocin is unusual in that the primary event in its mode of action is the immediate inhibition of RNA synthesis, while DNA synthesis is not affected and protein synthesis is inhibited after a delay of 20 min. No effect on intracellular ATP levels was observed.

5.1 INTRODUCTION

The adsorption of the bacteriocin molecules to the cell wall of a sensitive bacterium is usually demonstrated by mixing the bacteriocin with sensitive cells, at intervals samples are taken, diluted to prevent further appreciable adsorption of bacteriocin, and viable counts are carried out. The extent of killing by a bacteriocin depends primarily on the concentration of the bacteriocin and the "single hit" hypothesis was formulated (Reeves, 1972; Nomura, 1967). This hypothesis states that an adsorbed bacteriocin molecule has a certain probability of killing a sensitive cell, the probability being anything up to 1, depending on receptors on the cell wall of a sensitive bacterium (Nomura, 1967; Reeves, 1972). Most kinetic data on bacteriocins studied (Reeves, 1972; Nomura, 1967; and Tagg *et al.*, 1976) suggest that bacteriocins kill sensitive cells by single-hit kinetics. The stage which brings bacteriocins into effective contact with the cytoplasmic membrane may be the energy-dependent step which is inhibited by energy uncouplers. Furthermore, the

fact that most of the bacteriocin treated cells can be "rescued" by incubation with trypsin (Nomura, 1967; Tagg et al, 1976) is taken to support the theory that the killing action of a bacteriocin molecule adsorbed to the cell surface of a sensitive bacterium is divided into two stages. At stage I, the bacteriocin has adsorbed to the surface receptors but has not yet initiated a chain of events leading irreversibly to cell death. Cells at this stage can be "rescued" from the bacteriocin by incubation with trypsin, which is assumed to digest the bacteriocin molecule attached to the receptor sites. Stage II, however, is irreversible and is thought to occur when the bacteriocin or part of it has managed to penetrate the cell membrane of a susceptible organism, thereby initiating a chain of events leading to cell death. Uncouplers of oxidative phosphorylation, 2,4-dinitrophenol or p-trifluoromethoxycarbonyl cyanide phenylhydrazone, inhibit the transition from stage I to stage II (Plate & Luria, 1972) and it was therefore suggested that for the transition to stage II to occur the energised state of the cytoplasmic membrane was necessary (Jetten & Jetten, 1975).

The general biochemical effects of bacteriocins can be grouped into the following classes (Reeves, 1972):

Group 1: This group of bacteriocins affect the energy flux in sensitive bacteria. Usually, macromolecular synthesis is inhibited as a result of a rapid decrease in intracellular ATP levels. Examples of this group are colicins E, K, Ia and Ib (Reeves, 1972), morgacin 174 (Williams 1976a. There is also a decrease in ion transport across the membrane (Fields & Luria, 1969; Lusk & Nelson, 1972).

Group 2: Bacteriocins in this group primarily affect DNA metabolism. With colicin E2, not only is DNA synthesis inhibited but all pre-existing DNA is degraded. Colicin E2 has recently been

found to be a DNA endonuclease (Schaller & Nomura, 1976). Other bacteriocins which inhibit DNA synthesis are megacin C-C4 (Holland, 1965), pesticin (Elgat & Ben-Gurion, 1969).

Group 3: Bacteriocins in this group affect protein and RNA synthesis. The most studied is colicin E3 which inhibits protein synthesis by causing endonucleolytic scissions near the 3' terminus of the 16S rRNA of the 30S subunit of the ribosomes (Bowman et al., 1971; Senior & Holland, 1971). Colicin E3 has been shown to have intrinsic in vitro activity (Boon, 1972; Bowman et al., 1971) and most recently, Ohno & Imahori (1978) showed that highly purified colicin E3 is a RNA endonuclease. Another bacteriocin with a similar mode of action, i.e. inhibition of protein synthesis by an endonucleolytic scission near the 3' end of the 16S ribosomal RNA is cloacin DF13 (De Graaf et al., 1973). This bacteriocin has also been shown to have endonucleolytic action on Clo DF13 plasmid (Veltkamp et al., 1976).

Other bacteriocins in this group are pneumocins 196, S8 and S6 (De Graaf & Stouthamer, 1971), a bacteriocin from Clostridium septicum (Schallen & Krämer, 1976) and a bacteriocin from S. aureus.

Producer strains are usually immune to the action of the bacteriocin they produce. Immunity is probably the result of the synthesis of a specific substance (an "immunity substance") which neutralises the effect of the bacteriocin in the producer strain, or interferes with the transmission process in resistant strains (Nomura, 1963).

As there are no reports of the mode of action of a B. fragilis bacteriocin, it was decided to investigate the effect of the Bf-1 bacteriocin on macromolecular synthesis and intracellular ATP levels in B. fragilis.

5.2 METHODS

5.2.1 Media and Chemicals as described in Appendix.

5.2.2 Preparation of the Bf-1 bacteriocin

Two litres of supernatant fluid was prepared and sterilized over chloroform as described in Chapter 3. The supernatant was then partially purified and concentrated by precipitation with $(\text{NH}_4)_2 \text{SO}_4$. The 30 - 60% $(\text{NH}_4)_2 \text{SO}_4$ fraction was dialysed extensively against 0.02 M Tris-HCl buffer before use.

5.2.3 Killing units

The Survival Count Method described by Mayr-Harting et al., 1972, was used. Bacteriocin Bf-1 was added to exponentially growing indicator cells and allowed to adsorb by incubation at 37°C for 20 min. The cultures were then plated for survivors and the number of killing units per ml (K.U. ml^{-1}) was determined from the Poisson distribution

$$L_0 = -N_0 (\ln (N_\infty/N_0)) \quad \text{where}$$

L_0 = K.U. per ml sample; N_0 = viable cells per ml at time of mixing; and N_∞ = viable cells per ml at end of experiment.

5.2.4 Determination of protein and nucleic acid synthesis

Since the Bacteroides strains were unable to incorporate labelled amino acids (Burt & Woods, unpublished data), protein synthesis could not be determined by measuring the incorporation of either $\{^3\text{H}\}$ - or $\{^{14}\text{C}\}$ -labelled amino acids. The method of Elgat & Bengurion (1969) was used. Samples (9 ml) of culture were taken at different time intervals, mixed with 1 ml cold 50% TCA and left overnight at 4°C . The samples were then centrifuged, the pellets resuspended in 1.5 ml 5% TCA and extracted for 30 min at 90°C to obtain nucleic acid fractions. The supernatant contained

nucleic acids and the protein pellets were washed with 80% ethyl alcohol (5 ml) and boiled with 2 ml of 1N NaOH for 10 min. After centrifugation, protein was determined by the Lowry method (Lowry et al., 1951) and RNA by the orcinol method (Schneider, 1945). RNA and DNA synthesis were determined by difference after incorporation of [^{3} H]-adenine ($2 \mu\text{g ml}^{-1}$ $0.04 \mu\text{Ci ml}^{-1}$ final concentration). Total nucleic acid synthesis was determined by measuring the amount of TCA precipitable [^{3} H]-adenine at different time intervals. Samples (0.25 ml) were removed from cultures and added to 0.25 ml cold 10% TCA containing $1 \mu\text{g ml}^{-1}$ cold adenine and kept on ice for 30 min. They were then filtered on Whatman GFA filters and the precipitates washed twice with 10 ml cold 5% TCA and once with 10 ml 1% acetic acid. The filters were dried and counted.

To determine DNA synthesis, samples (0.25 ml) were withdrawn and added to 0.25 ml 1N NaOH and incubated at 37°C overnight to hydrolyze both RNA and protein. The resulting samples were then added to 0.5 ml cold 10% TCA and kept on ice for 30 min before filtering. The pellets were washed as described above and filters dried and counted. RNA synthesis was estimated from the difference between the total nucleic acid and the DNA synthesis counts.

5.2.5 Estimation of intracellular levels of ATP

Intracellular ATP levels were determined 10 min after the addition of bacteriocin or after cultures had been vigorously aerated for 10 min and compared with the relevant controls. The dried firefly extracts were rehydrated to a concentration of 10 mg ml^{-1} in 0.05 M potassium arsenate (pH 7.4) and 0.02 M magnesium sulphate. The extract was used within 3 h after it had been rehydrated.

ATP was extracted from 0.5 ml samples of culture by boiling with 4.5 ml of distilled water at 100°C for 10 min as described by Fields & Luria (1969).

The assays were performed by first mixing the sample (5.0 ml) in a scintillation vial with 2.4 ml buffer (0.05 M potassium arsenate, 0.02 M MgSO_4 , 0.001 M EDTA pH 7.4). At zero time, 0.5 ml of firefly lantern extract at 25°C was added, and the contents in the vial mixed. The vial was then lowered into the counting chamber of a Beckman model LS 3150T liquid scintillation counter and the light flashes emitted counted. The whole procedure did not take more than 6 sec. The amount of ATP in the sample was estimated from counts obtained by adding known amounts of ATP to buffer. ATP levels in the supernatant were estimated by removal of cells by centrifugation in a Beckman Microfuge. These levels were negligible, both in the control and bacteriocin-treated cultures.

5.3 RESULTS

5.3.1 Killing kinetics

Addition of bacteriocin (4 killing units) to exponentially growing indicator (Bf-2) cells caused a rapid decrease in viability (Fig 5.1). The killing kinetics were biphasic and a resistant population was selected after 20 min. The action of the bacteriocin showed single-hit kinetics when exponentially growing Bf-2 cells were treated with various bacteriocin concentrations at 37°C for 20 min (Fig 5.2). The turbidity of a sensitive culture treated with bacteriocin remained constant over 60 min indicating that the action of the bacteriocin is bactericidal but not bacteriolytic. The killing of sensitive cells on plates was markedly affected by temperature (Fig 3.3a). Sensitive cells treated with bacteriocin (64 A.U.) for 30 min at 42, 37, 30 and 24°C showed 10, 51, 100 and 100% survival respectively (Fig 5.3b). The effect of temperature on bacteriocin

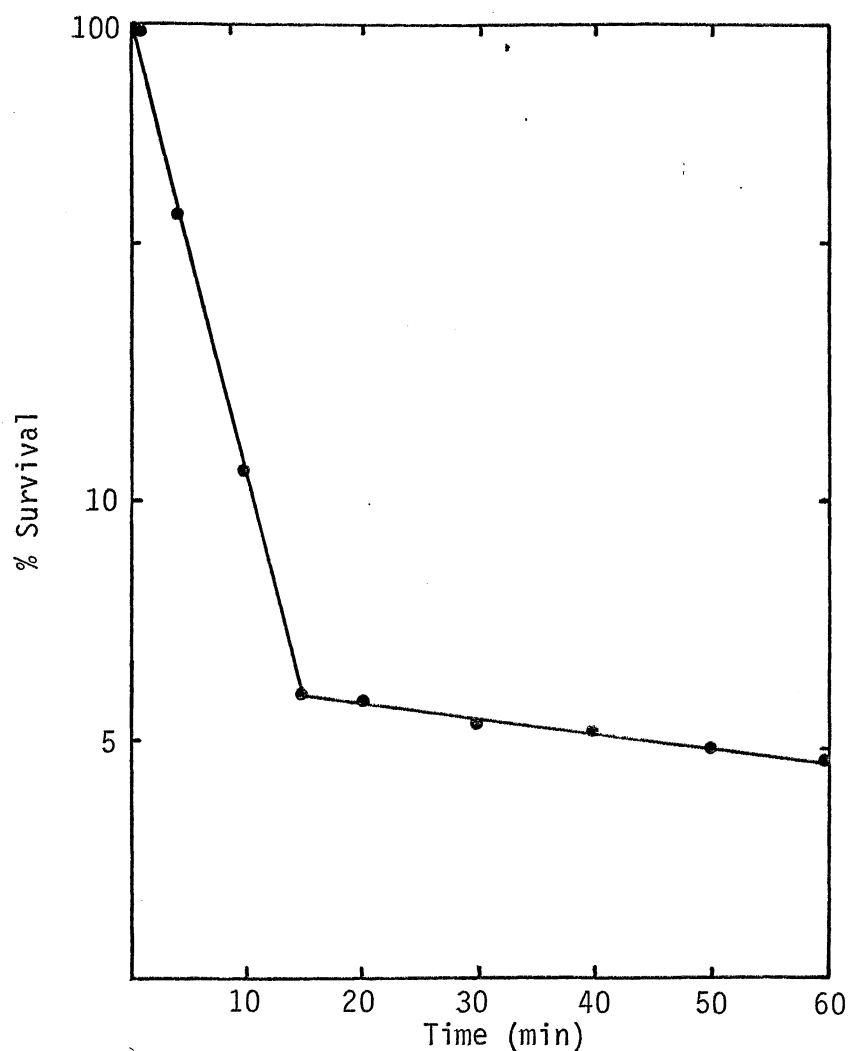


Fig. 5.1 Kinetics of bacteriocin and induced lethality. Exponentially growing *Bacteroides* cells were treated with 4 killing units of bacteriocin and plated for survivors.

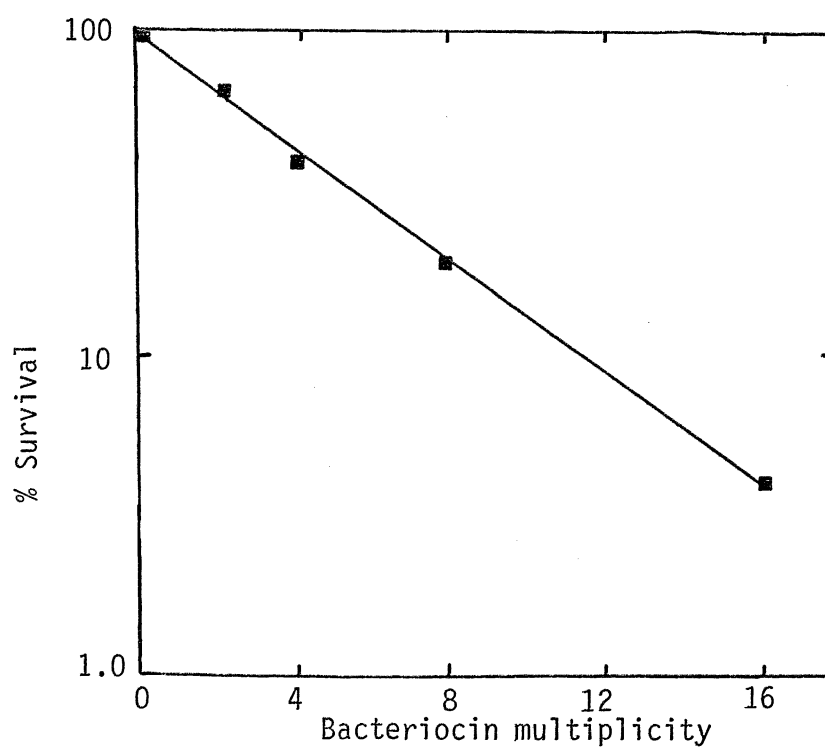
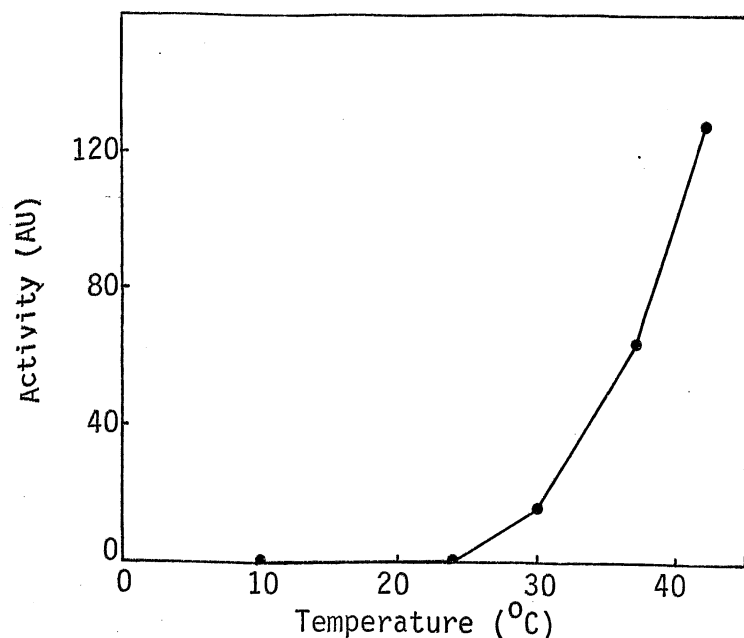


Fig. 5.2 Single-hit kinetics of bacteriocin action. Exponentially growing *Bacteroides* cells were treated with various bacteriocin concentrations at 37°C for 20 min. The bacteriocin concentration was expressed as a multiplicity of 16 = 128 AU and a multiplicity of 2 = 16 AU.



(b) Broth

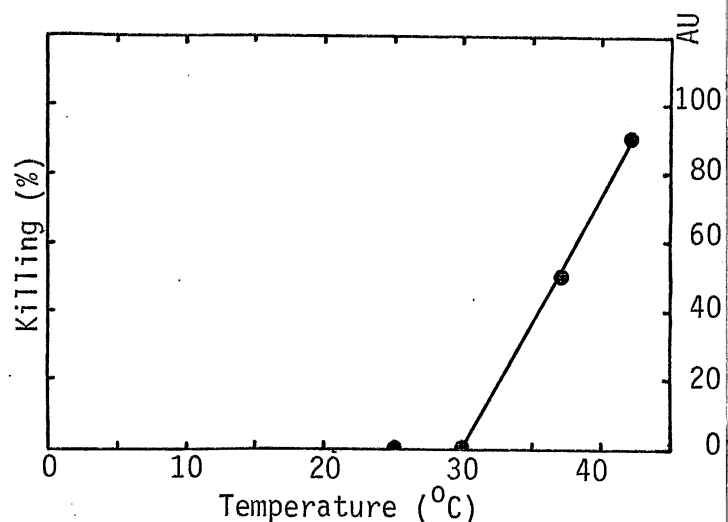


Fig. 5.3 Effect of temperature on Bf-1 bacteriocin action.

(a) Bacteriocin sample assayed for activity at different temperatures on seeded plates containing sensitive Bf-2 indicator strain.

(b) Added to log phase Bf-2 cells, held at different temperatures for 30 min before plating for survivors.

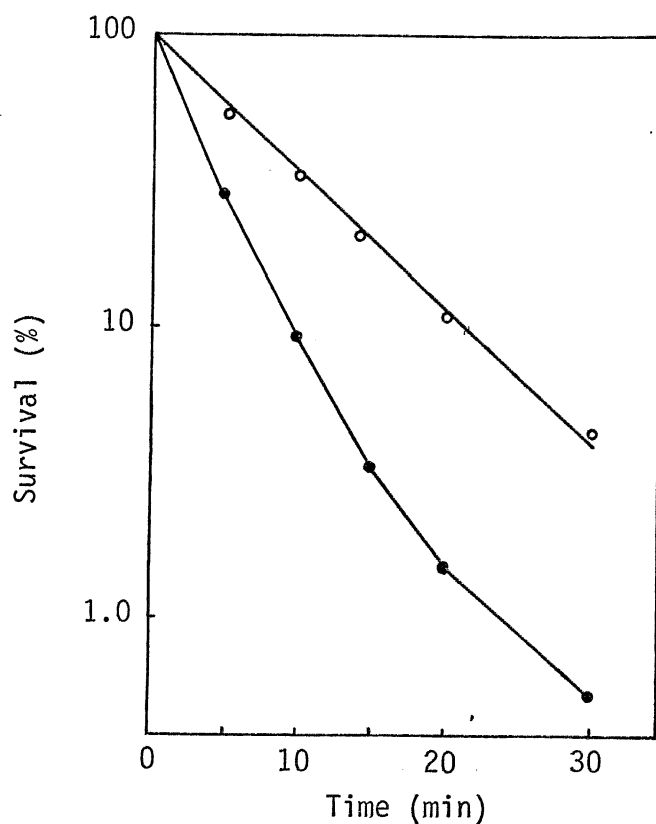


Fig. 5.4

Effect of growth phase on Bf-1 bacteriocin action. Cells were treated with Bf-1 bacteriocin (4 KU) and plated as described in Fig. 5.1. (●) Exponential phase cells; (○) Stationary phase cells.

adsorption was determined by incubating cells and bacteriocin at 20 and 37°C for 30 min. The cells were removed by centrifugation and the concentration of the bacteriocin in the supernatants was estimated by treating fresh cells for 20 min at 37°C and plating for survivors. After adsorption at 20 and 37°C incubating of fresh cells resulted in 11 and 33% survival respectively. Exponential phase cells were more sensitive to killing by the bacteriocin than stationary phase cells (Fig 5.4).

5.3.2 Trypsin rescue of bacteriocin treated cells

Studies on the effect of treatment of sensitive cells with trypsin, 2,4-dinitrophenol (DNP) and sodium arsenate indicated that the bacteriocin-treated cells were trypsin rescuable, and that the interaction between the bacteriocin and the sensitive cells occurred in two stages (Fig.5.5). There is a stage after adsorption of the bacteriocin when the cells are trypsin rescuable and physiologically unaffected, followed by a transition to a second stage when the cells are physiologically damaged and trypsin rescuable (results not shown but the transition from stage I to stage II also depends on the concentration of the bacteriocin). Treatment with trypsin did not overcome the RNA inhibition in bacteriocin treated cells after transition to stage II. Pretreatment of cells with DNP resulted in all the cells remaining in stage I and hence trypsin rescuable.

5.3.3 Effect on protein and nucleic acid synthesis

Since the *Bacteroides* strain did not incorporate labelled amino acids, protein synthesis was determined by measuring the protein concentration of TCA-precipitable material (Fig 5.6). In order to obtain a comparison between RNA synthesis and protein synthesis, RNA synthesis was also determined in the same experiment by an analogous method. The results indicate that in cells treated with 64 AU bacteriocin, there was a rapid and complete inhibition in RNA synthesis but that protein synthesis

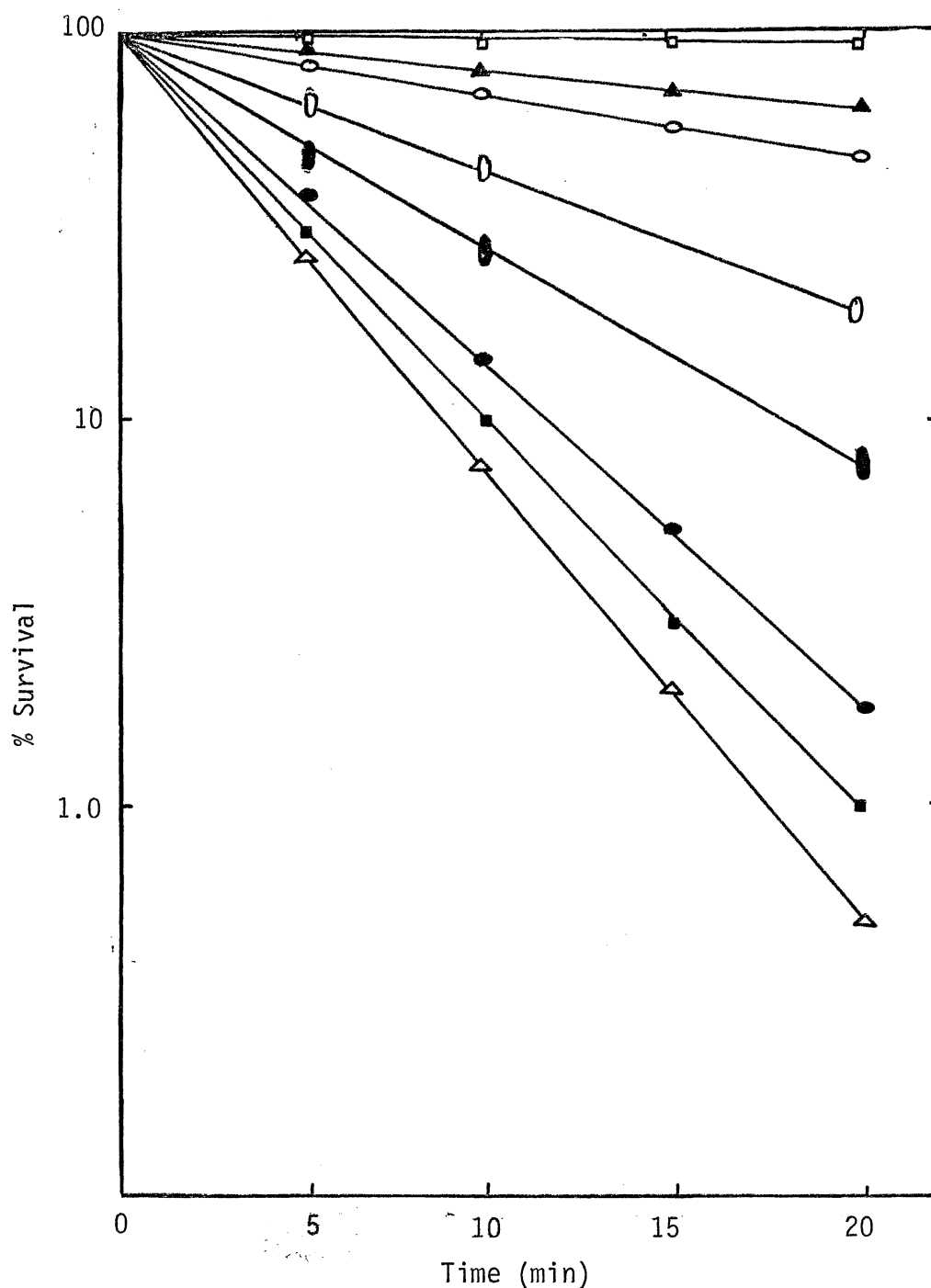


Fig. 5.5 Trypsin rescue of bacteriocin treated cells. Exponentially growing *Bacteroides* cells were treated with bacteriocin (4 killing units/colony forming unit). At intervals samples were plated for survivors (■) or diluted into trypsin (5 mg ml⁻¹ in 20 mM Tris-HCl, pH 8) and held for 30 min at 37°C before plating (○). Cells pretreated with trypsin for 30 min at 37°C before exposure to bacteriocin (△). Cells pretreated with 20 mM 2,4-dinitrophenol for 30 min at 37°C before exposure to bacteriocin (○) and rescued with trypsin (□) or 2,4-dinitrophenol removed before treatment with bacteriocin (●). Cells pretreated with 100 mM sodium arsenate for 20 min at 37°C before exposure to bacteriocin (◆) and after trypsin rescue (▲).

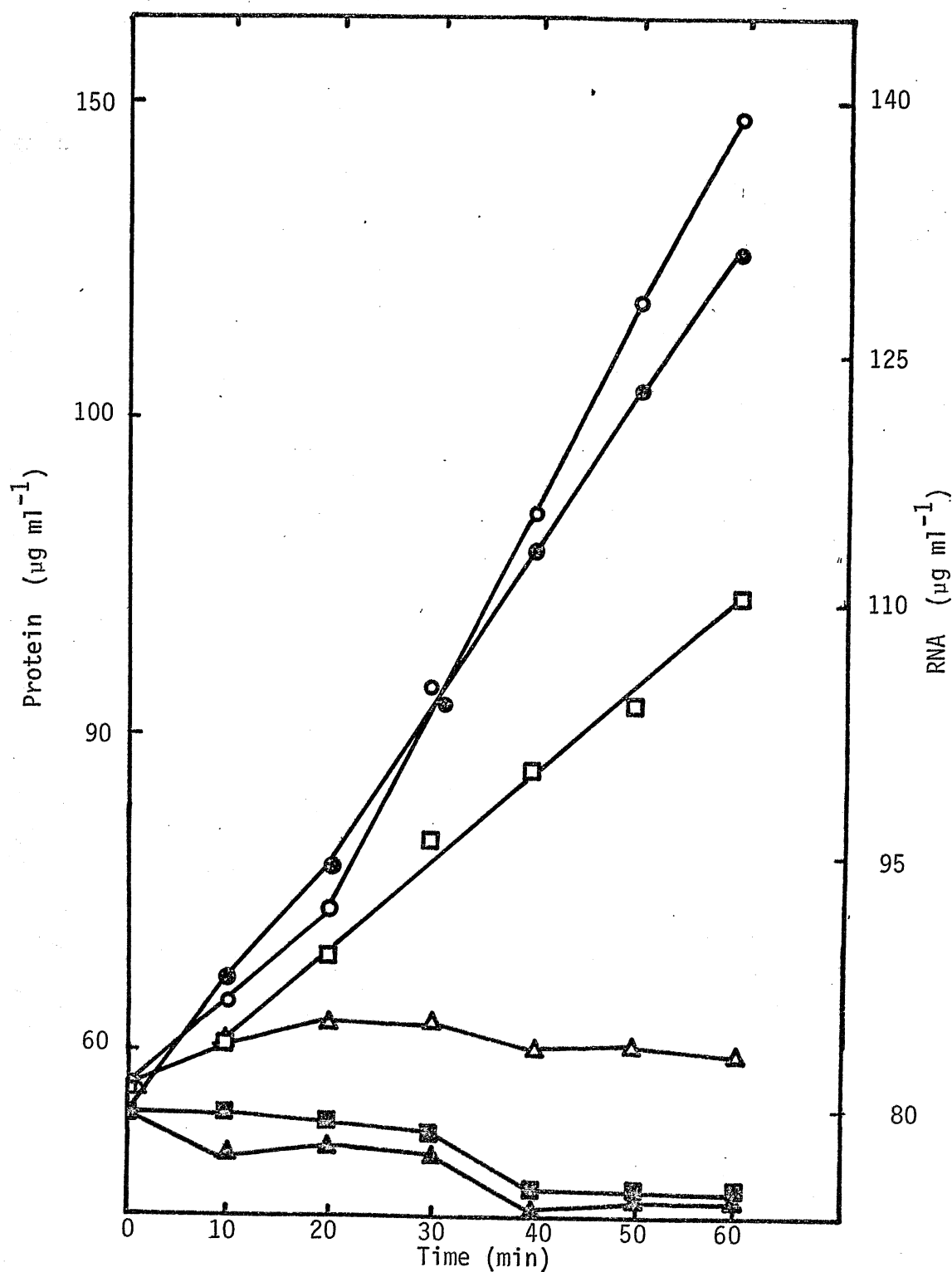


Fig. 5.6 Effect of the bacteriocin on protein and RNA synthesis. Bacteriocin was added to exponential cultures at time zero. Protein synthesis control culture (○); with 64 AU bacteriocin (□); with 256 AU bacteriocin (△). RNA synthesis control culture (●); with 64 AU bacteriocin (■); with 256 AU bacteriocin (▲).

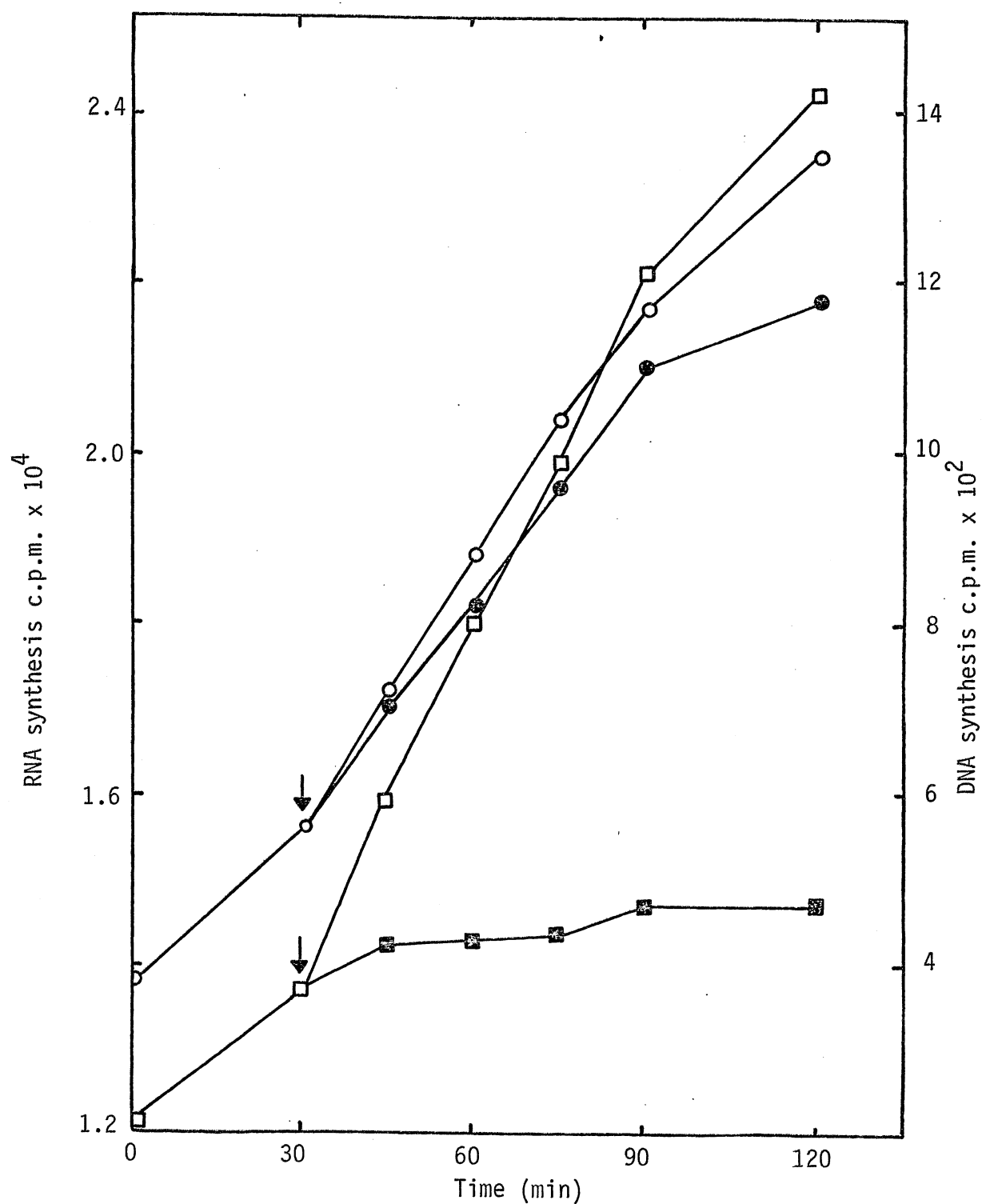
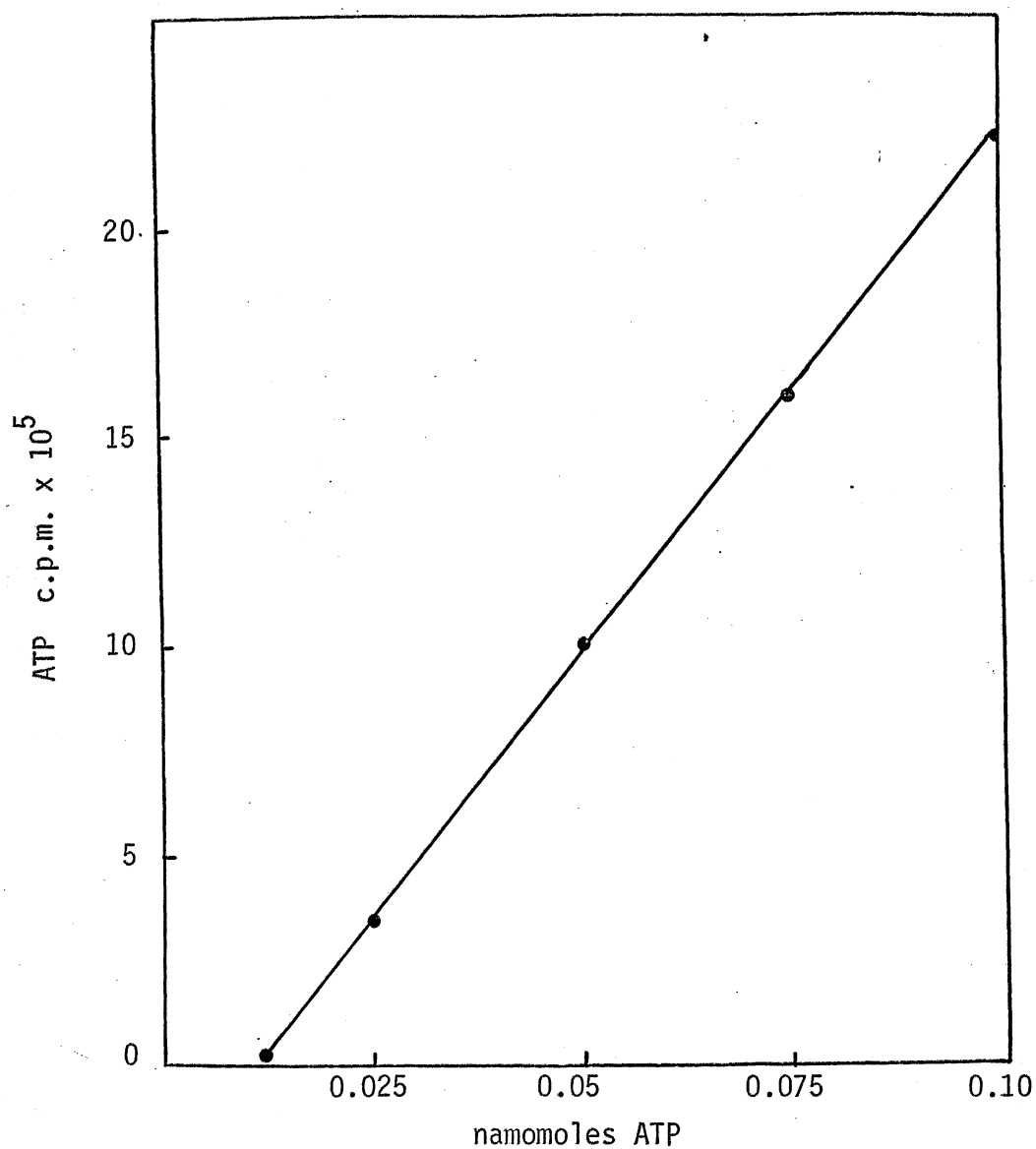


Fig. 5.7 Effect of the bacteriocin on DNA and RNA synthesis. Exponential cultures were grown for 30 min before the addition of bacteriocin (64 AU) (arrow). DNA synthesis control culture (○); with bacteriocin (●). RNA synthesis control culture (□); with bacteriocin (■). Time of addition of bacteriocin ↓.



ATP standard curve. Samples (0.5 ml) of ATP stock solution at different concentrations counted as described in Methods.

Table 5.1 Intracellular ATP levels

Time (min)	Anaerobic		Aerobic Control
	Control	+ Bacteriocin	
0	100%	100%	100
10	100%	97%	63%

was only partially inhibited after 20 min. At higher concentrations of the bacteriocin (256 AU) the inhibition of protein synthesis was more marked but this inhibition still lagged behind the inhibition of RNA synthesis. Studies on the effect of bacteriocin on DNA and RNA synthesis in sensitive cells, estimated by the incorporation of labelled $\{^3\text{H}\}$ thymidine into TCA-precipitable material showed that DNA synthesis was not affected but RNA synthesis was inhibited (Fig 5.7). The 3% heat-stable fraction after autoclaving had the same effect on macromolecular synthesis as the untreated bacteriocin.

5.3.4 Effect on intracellular ATP

The bacteriocin had no effect on intracellular ATP levels. Ten minutes after the addition of bacteriocin the level of ATP was 97% of that of untreated control cultures (Table 1(a)). When the Bacteroides strain was aerated for 10 min in the absence of the bacteriocin the ATP levels fell to 63% of that of anaerobic control cultures (Table 5.1). Aeration of the anaerobe Clostridium acetobutylicum is known to result in a marked decrease in intracellular ATP (O'Brein & Morris, 1971).

5.4 DISCUSSION

The killing action of the Bf-1 bacteriocin follows single-hit kinetics, and, as with many other bacteriocins, a single bacteriocin molecule has a certain probability of causing the death of a sensitive cell. This lethal action is dependent both on the temperature of incubation and the growth phase of the sensitive cell. This suggests that the adsorption of the bacteriocin to the sensitive cell and the

subsequent transmission of its lethal action requires actively growing cells. This prerequisite has also been shown for other bacteriocins (Basinger & Jackson, 1968; Jetten & Vogels, 1974; Holland, 1962; Mahoney & Butler, 1971).

It is concluded that the action of the Bf-1 bacteriocin occurs in 2 stages since bacteriocin-treated cells can be rescued by trypsin. The temperature of incubation is also important in regulating the number of killing units of a bacteriocin sample. Furthermore, the fact that dinitrophenol-treated cells were mostly trypsin rescuable indicates that the transition from stage I to stage II is energy dependent.

The mode of action of the Bf-1 bacteriocin is somewhat unusual and, as a result, cannot be placed with any of the bacteriocins studied to date. The Bf-1 bacteriocin completely inhibited RNA synthesis while having no effect on DNA synthesis or intracellular ATP levels. At low bacteriocin concentrations, protein synthesis was partially inhibited after a delay. It is concluded that the primary event in the mode of action of the Bf-1 bacteriocin is the inhibition of RNA synthesis. At higher bacteriocin concentrations the inhibition of RNA synthesis is followed by the inhibition of protein synthesis. The inhibition of protein synthesis is presumably as a result of the inhibition in RNA synthesis. The mode of action of the Bf-1 bacteriocin is most interesting in that while it specifically inhibits RNA synthesis, it has no effect on DNA synthesis and a delayed effect on protein synthesis. Bacteriocins which have closely related modes of action on sensitive cells are pneumocin G 196 (De Graaf & Stouthamer, 1969) and a bacteriocin from Clostridium septicum (Schallen & Krämer, 1976) which inhibit protein and RNA synthesis whilst having little or no effect on DNA synthesis. This biochemical effect is different from that of other colicins studied

and shown to affect either DNA synthesis (endonuclease activity) (Schaller & Nomura, 1976), protein synthesis (endonucleolytic ribosomal RNA cleavage) (Bowman et al., 1971), or energy metabolism (Feingold, 1970; Hirata et al., 1969; Williams & Krizsanovich-Williams, 1977). The Bacteroides bacteriocin may be useful as a specific inhibitor of RNA synthesis in studies on transcription in this important anaerobe.

CHAPTER VI

RIFAMPICIN-RESISTANT Bf-2 MUTANTS AND THEIR TOLERANCE TO THE BACTERIOCIN PRODUCED BY Bf-1

6.1 INTRODUCTION

Since the mode of action of the Bf-1 bacteriocin appeared to be specific for RNA synthesis, the comparison of bacteriocin resistance with resistance to other compounds which are known to inhibit RNA synthesis would be of interest. Among the well known and much studied inhibitors of transcription are rifampicin (rif) and streptolydigin (std). Rif is the most studied antibiotic affecting RNA synthesis. It is a specific inhibitor of bacterial RNA polymerase (Hartman et al., 1967) which, unlike other compounds that interfere with transcription (Kersten & Kersten, 1974) acts by direct interaction with the enzyme, blocking the initiation, but not the elongation, of RNA chains (Sippel & Hartman, 1968). It has been suggested that rifampicin acts by either preventing

- (a) the binding and stabilization effect of the initiating purine triphosphate (di'Mauro et al., 1969);
- (b) the formation of an active site of the binary complex required for initiation (Sippel & Hartman, 1970);
- (c) the formation of the first phosphodiester bond (So & Downey, 1970); or
- (d) the formation of the second phosphodiester bond (Johnson & McClure, 1976).

Since mutations mapping at the rif locus (within the structural gene for the β -subunit of RNA polymerase; Heil & Zillig, 1970) of the E. coli

chromosome resulted in altered RNA polymerases (Miller et al., 1976) which were resistant to the antibiotic rifampicin (Tocchini-Valentini et al., 1968), the initial aim in this chapter was to isolate such mutants and determine whether rifampicin resistance could be correlated to bacteriocin resistance.

Evidence of altered RNA polymerases has been well documented in previous studies (Claeys et al., 1969; Ishihama, 1976; Kirschbaum et al., 1975). Moreover, strong evidence suggests that genes coding for rifampicin (rif) and streptolydigin (std) sensitivities determine components of both vegetative and sporulation RNA polymerases in Bacillus subtilis (Losick & Sonenshein, 1969; Sonenshein et al., 1974) and furthermore, the finding that rif^R and std^R mutants have reduced sporulation abilities supported this notion (Doi et al., 1970; Bonamy et al., 1973; Sonenshein & Losick, 1970; Leighton, 1973).

There are three known "biochemical mechanisms" by which bacterial cells can become insensitive to bacteriocins (Nomura, 1967; Reeves, 1972). These are resistance, tolerance and immunity. Resistance to any type of bacteriocin occurs when a cell fails to absorb a type of bacteriocin, presumably owing to a lack of a relevant receptor. Most spontaneous mutations resulting in insensitivity to bacteriocins are due to receptor site mutations. Since most surface receptors are specific for different bacteriocins, most bacterial strains are resistant to other bacteriocins produced by unrelated strains and this type of resistance does not necessarily have to involve a mutation.

Bacteriocinogenic cells are insensitive to the bacteriocin they produce although they retain the ability to adsorb it (Maeda & Nomura, 1966a). This insensitivity is called immunity. The nature of the immunity is still generally unknown although evidence strongly implicates the action of an

inhibitor of bacteriocin action (Bowman et al., 1971, Ochi et al., 1970; Jakes, Zinder & Boon, 1974). This inhibition appears to be produced in equal molar amounts with the bacteriocin and is, in most cases, acidic. The exception to this statement is called immunity breakdown and only occurs when very large amounts of homologous bacteriocin are added to the producer culture.

A class of mutants has been isolated which, though retaining the ability to adsorb the bacteriocin, are still refractory to their action. The mutants are called tolerant mutants and Reeves (1972) recognized 5 classes of tolerance col mutants. It has been suggested (Nagel de Zwaig & Luria, 1967; Rolfe & Onadera, 1972) that a tolerant mutation involves alterations in the cell membrane and that the adsorbed bacteriocin somehow fails to act on the target inside the cell. Nomura (1967) classified tolerant mutants into 3 groups:

- (a) one which fails to initiate the proposed stimulus at the receptor site;
- (b) one which has some defect in the proposed transmission mechanism;
- (c) one which has some alteration in the target itself. These alterations can be induced directly or indirectly (Nomura, 1967).

In our attempt to compare rif-resistance and resistance to the Bf-1 bacteriocin, one is dealing with type (c) tolerance above. We therefore isolated rif^R mutants and determined whether they were also resistant to the Bf-1 bacteriocin. These strains were further characterized by assaying directly for RNA polymerase and determining the effect of the Bf-1 bacteriocin and rifampicin on WT Bf-2 and its rif^R mutants. Furthermore, we also tested the immunity conferred on Bf-1 in vitro using the same system.

6.2 METHODS

6.2.1 Isolation of rifampicin-resistant mutants

BHI-agar gradient plates containing 0 - 20 $\mu\text{g rif ml}^{-1}$ were prepared. Overnight cultures of the bacteriocin-sensitive strain Bf-2 were spread onto the gradient plates containing rif. The plates were then incubated for 24 h. Colonies showing slight resistance to rif were picked and streaked onto fresh gradient plates. This procedure was repeated until growth of Bf-2 covered the whole plate. Isolated colonies were picked and tested for resistance to rif and compared with the control rif^S strain. Rif^R mutant strains were isolated and assayed for resistance to the Bf-1 bacteriocin.

6.2.2 In vivo RNA synthesis

RNA synthesis in the presence of rif was monitored for both WT Bf-2 and the rif^R mutants. Cells were grown in BHI-broth until they reached mid-log phase (absorbance ₆₆₀ ca. 0.60). The cultures were pelleted by centrifugation, washed and resuspended in glucose minimal medium (Varel & Bryant, 1974), adjusted to pH 7.7, at a starting concentration of ca. $A_{660} = 0.1$. Cultures were allowed to grow at 37°C to A_{660} ca. 0.2 before the addition of [³H]-uracil (0.4 $\mu\text{Ci ml}^{-1}$ final concentration). Samples (0.1 ml) were taken at intervals and the amount of TCA-precipitable material determined by the addition of samples to 0.1 ml cold 10% TCA containing 1 $\mu\text{g ml}^{-1}$ cold uracil. The samples were then allowed to precipitate by leaving them in ice for at least 30 min before filtering on Whatman glass fiber (GFA) discs, the precipitates were washed twice with 10 ml 5% TCA (cold) and once in 1% acetic acid. The discs were dried at 50°C for 3 h before being counted. Rifampicin (25 $\mu\text{g ml}^{-1}$) was also added to some cultures and the amount of [³H]-uracil incorporation determined as described above.

6.2.3 In vitro RNA synthesis

Exponential phase cultures (200 ml) of the producer strain (Bf-1), the sensitive indicator (Bf-2) strain and mutants (rif^R) of Bf-2 (i.e. Bf12 x 2, Bf-2 x 13 and Bf-2 x 14) grown in BHI-broth were harvested by centrifugation. The pellets were washed twice before being resuspended in a buffer solution (3 ml) containing 0.05 M Tris-HCl, $\frac{3}{4}$ 1 mM EDTA, 0.1 mM dithiothreitol and 10% (v/v) glycerol. The cells were disintegrated by sonication using an MSE sonicator at 8 microns for 4 min at 4°C in the above buffer solution containing in addition 0.5 mM phenylmethylsulphonyl fluoride before being centrifuged at 24 000 g for 15 min at 4°C. The supernatant fluid (0.1 ml) was assayed for RNA polymerase activity. The assay mixture contained 4 mM Tris-HCl pH 7.9, 0.4 mM MgCl₂, 0.1 mM Mn Cl₂, 0.015 M KCl, 1.2 mM β-mercaptoethanol and 0.4 mM ATP, CTP, GTP and [³H]-UTP (0.4 μCi ml⁻¹) and 250 μg ml⁻¹ Salmon Sperm DNA. Background counts were determined by omitting GTP from the assay mixture. The effects of rif (μg ml⁻¹ final concentration) and the Bf-1 bacteriocin (64 AU) were determined by adding 0.1 ml of each and RNA polymerase activity assayed at different time intervals.

The assay was initiated by the addition of [³H]-UTP (0.4 μCi ml⁻¹) 4 mM to the assay mixtures at 37°C. Samples (0.1 ml) were withdrawn at different time intervals, added to 10% TCA (cold) containing 1 μg ml⁻¹ cold UTP, kept on ice for at least 30 min before filtering on Whatman GFA filter paper discs. The discs were washed, dried, and counted as described above.

6.3 RESULTS

6.3.1 Isolation of Rif^R mutants

Rif^R mutants of the wild-type indicator strain, Bf-2, were easily

isolated after about 5 cycles using the isolation method described above. Eleven potential rif^R mutant strains were selected, tested for their resistance to rifampicin (Table 6.1) and stored for further study.

Table 6.1 Rif Minimal Inhibitory Concentration of WT and Rif^R strains

Strain		<u>Rif</u> MIC ($\mu\text{g ml}^{-1}$)	Phenotype
Bf-1	(WT)	<4 $\mu\text{g ml}^{-1}$	Sensitive
Bf-2	(WT)	<4	"
Bf-2 x 1	(<u>Rif</u> ^R)	>30	"
Bf-2 x 2	"	>30	"
Bf-2 x 3	"	"	"
Bf-2 x 4	"	"	"
Bf-2 x 5	"	"	"
Bf-2 x 6	"	"	"
Bf-2 x 7	"	"	"
Bf-2 x 13	"	"	"
Bf-2 x 14	"	"	"
Bf-2 x 15	"	"	"

6.3.2 Bacteriocin Insensitivity

The insensitivity of the rif^R mutants to the Bf-1 bacteriocin treatment in vivo was demonstrated by the normal assay method for bacteriocin. When the rif^R mutants were tested for their sensitivity to the Bf-1 bacteriocin (64 AU), 10 of the 11 mutant strains were found to be insensitive to varying concentrations of the bacteriocin (Table 6.2). The eleventh

strain (Bf-2 x 14), was, however, interesting in that it was more sensitive than the wild-type Bf-2 indicator strain although it still retained resistance to rif. In fact, the zones obtained when mutant strain Bf-2 x 14 was treated with the Bf-1 bacteriocin are interesting in that they are a mixture of class I (clear zones) and class II (turbid) zones, which is not normally observed with bacteriocin inhibition.

Table 6.2 Effect of the Bf-1 bacteriocin on isolated rif^R strains

Strain	Bacteriocin titre	Observations	Bacteriocinogenic Phenotype
Bf-2	64	clear zones	WT sensitive
Bf-2 x 1	1	turbid zones	<u>rif</u> ^R resistant
Bf-2 x 2	1	turbid zones	<u>rif</u> ^R resistant
Bf-2 x 3	4	clear zones	<u>rif</u> ^R resistant
Bf-2 x 4	1	clear zones	<u>rif</u> ^R resistant
Bf-2 x 5	16	clear zones	<u>rif</u> ^R resistant
Bf-2 x 6	4	clear zones	<u>rif</u> ^R resistant
Bf-2 x 7	2	clear zones	<u>rif</u> ^R resistant
Bf-2 x 13	16	turbid zones	<u>rif</u> ^R resistant
Bf-2 x 14	128	clear with large turbid boundary	<u>rif</u> ^R supersensitive
Bf-2 x 15	2	clear zones	<u>rif</u> ^R resistant

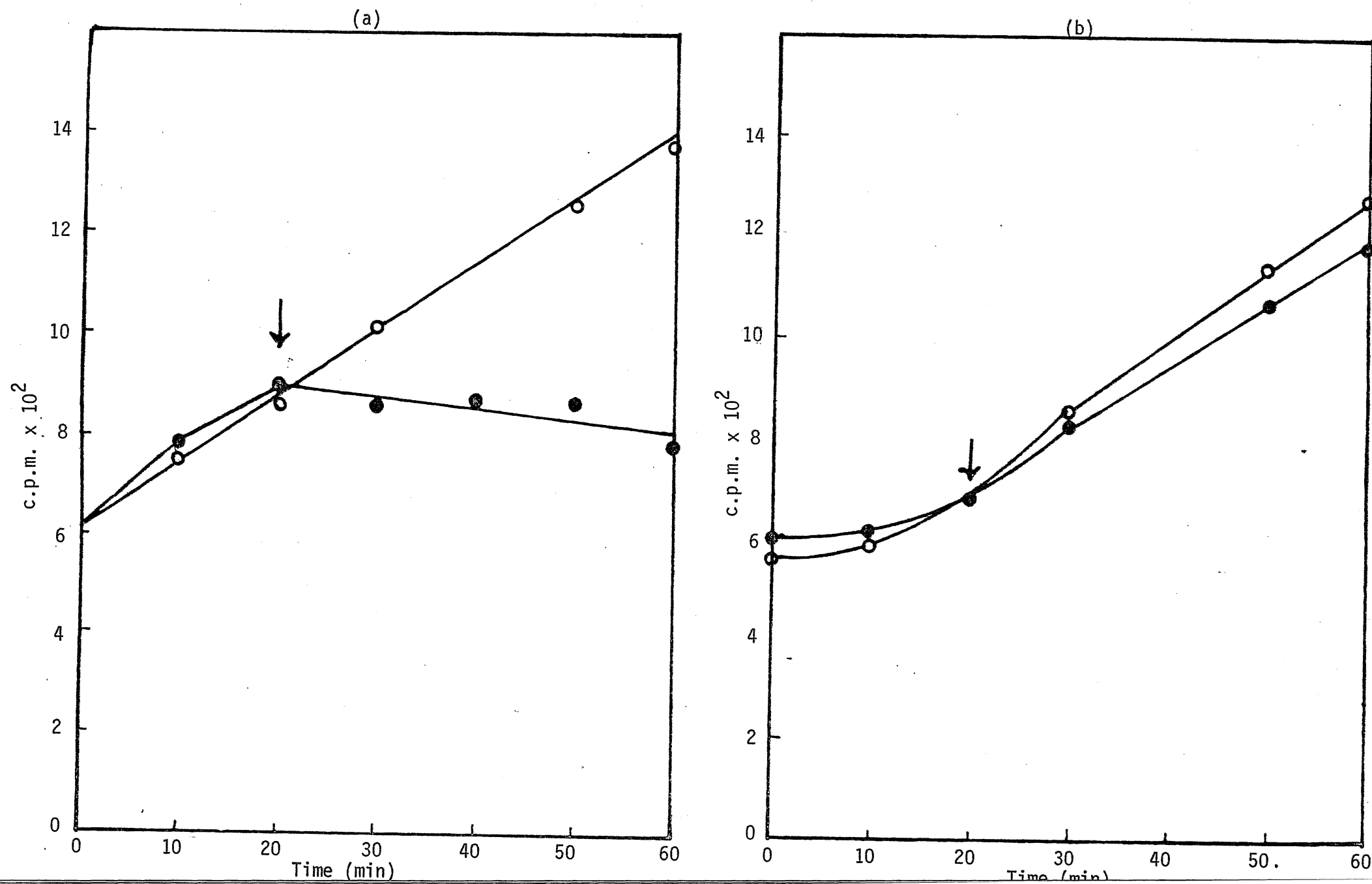
6.3.3 In vivo RNA synthesis

Addition of rifampicin ($25 \mu\text{g ml}^{-1}$) 20 min after the addition of labelled [^3H] uracil resulted in immediate inhibition of RNA synthesis in wild-type Bf-2 strain (Fig 6.1(a)). Rif^R mutant strains (Bf-2 x 2, Bf-2 x 13 and Bf-2 x 14) showed no such inhibition of RNA synthesis and a typical profile of RNA synthesis for Rif^R mutants is shown in Fig 6.1(b) for Bf-2 x 2.

6.3.4 In vitro RNA Polymerase assay

The effects of rifampicin ($25 \mu\text{g ml}^{-1}$) and Bf-1 bacteriocin (64 AU) on DNA-dependent RNA polymerases of the strains Bf-1, Bf-2, Bf-2 x 2, Bf-2 x 13 and Bf-2 x 14 are shown in Figs 6.2(a) to 6.2(e). While the rif^R mutants, Bf-2 x 2, Bf-2 x 13 and Bf-2 x 14 had enzymes resistant to rif (shown by increase in RNA synthesis even when rif is added) (Figs 6.2(c), (d) and (e)), wild-type producer (Bf-1) and sensitive (Bf-2) strains were inhibited in their in vitro RNA synthesizing abilities by rif (Fig 6.2(a) and (b)). Addition of 0.1 ml of the Bf-1 bacteriocin to the different assay mixtures again indicated that the rif^R mutants Bf-2 x 2 and Bf-2 x 13 were resistant to the Bf-1 bacteriocin while wild-type Bf-2 was inhibited. Again, the degree of inhibition of RNA polymerase activity by the Bf-1 bacteriocin in rif^R strain Bf-2 x 14 was more marked than in wild-type Bf-2. The bacteriocin producing strain (Bf-1), was not affected by its own bacteriocin although RNA synthesis was repressed, in vitro, by the addition of larger amounts (128, 256 and 512 AU) of the bacteriocin (Fig 6.2(b)). This phenomenon strongly resembles immunity breakdown.

Fig 6.1 Effect of rifampicin on RNA synthesis in log phase Bf-2 WT and *rif^R* cultures. Incorporation of ³H-uracil into TCA precipitable material. (a) WT Bf-2 cells with (●) and without (○) rifampicin (b) *rif^R* mutant Bf- 2 x 2 with (●) and without (○) rifampicin. ↓, time of addition of 25 μg ml⁻¹ rifampicin.



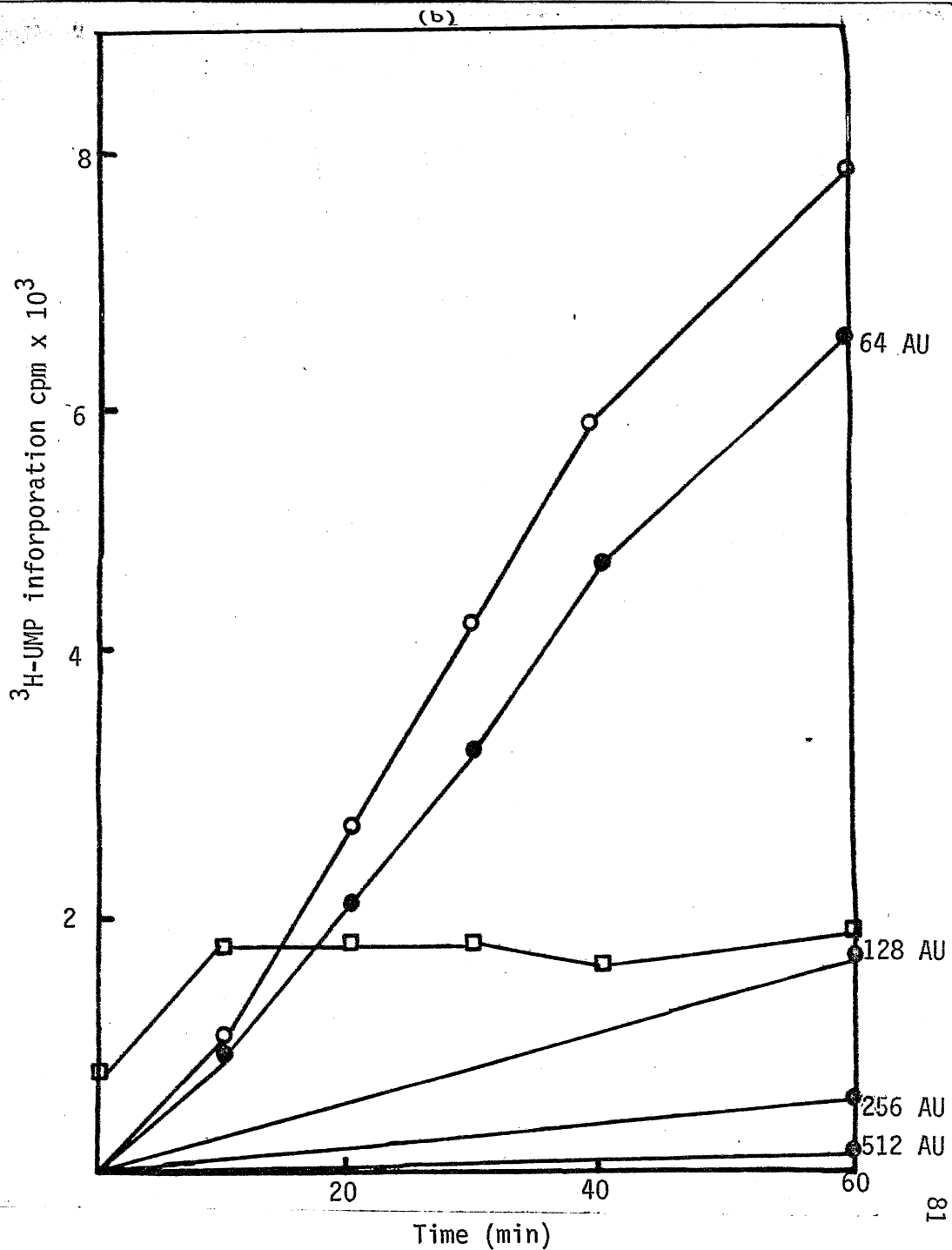
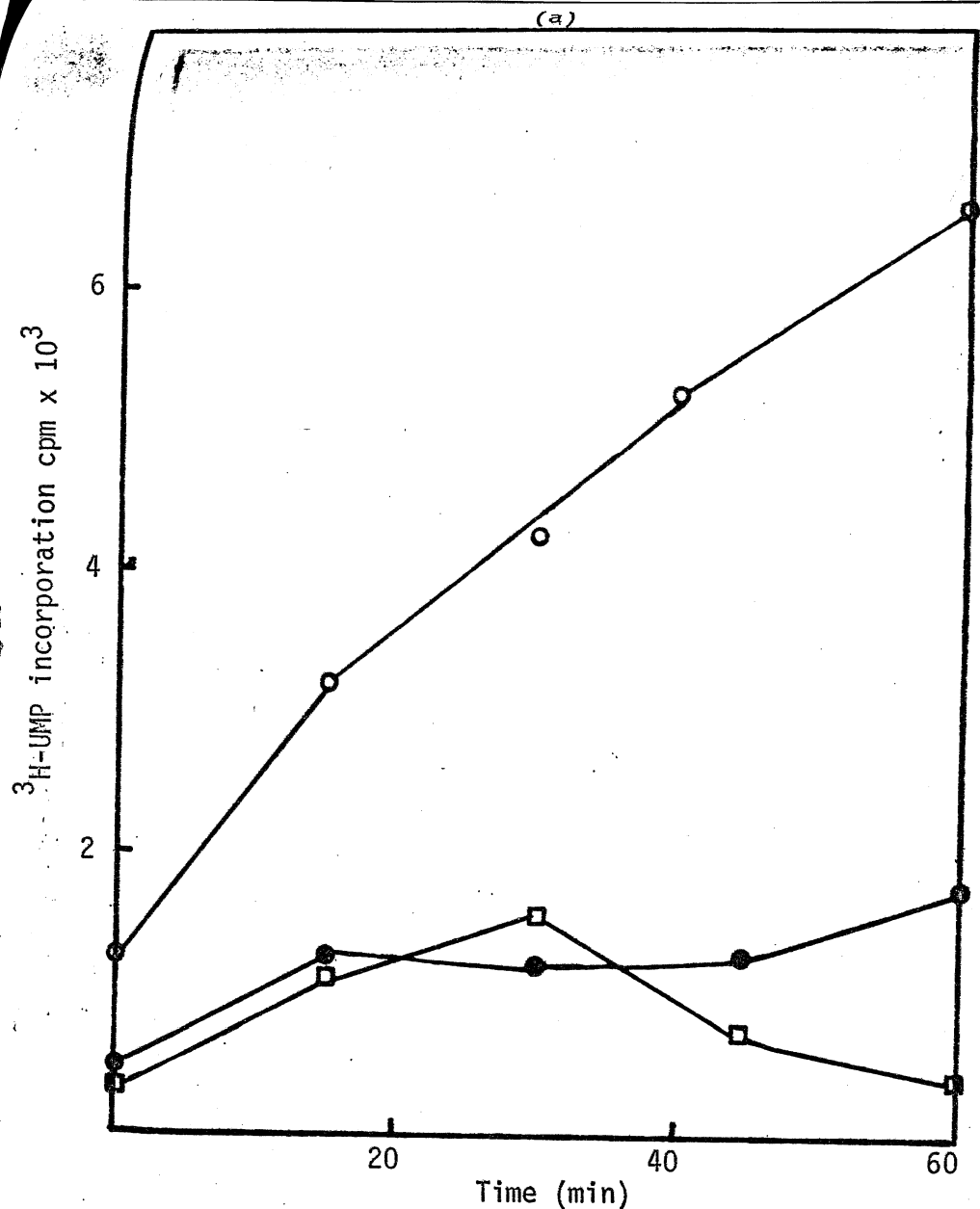


Fig 6.2 Effect of rifampicin (□) ($25 \mu\text{g ml}^{-1}$) and the Bf-1 Bacteriocin (64 AU) (●) on RNA polymerase activity. (a) WT-sensitive Bf-2, (b) WT bacteriocin producer strain (Bf-1), (c) Control

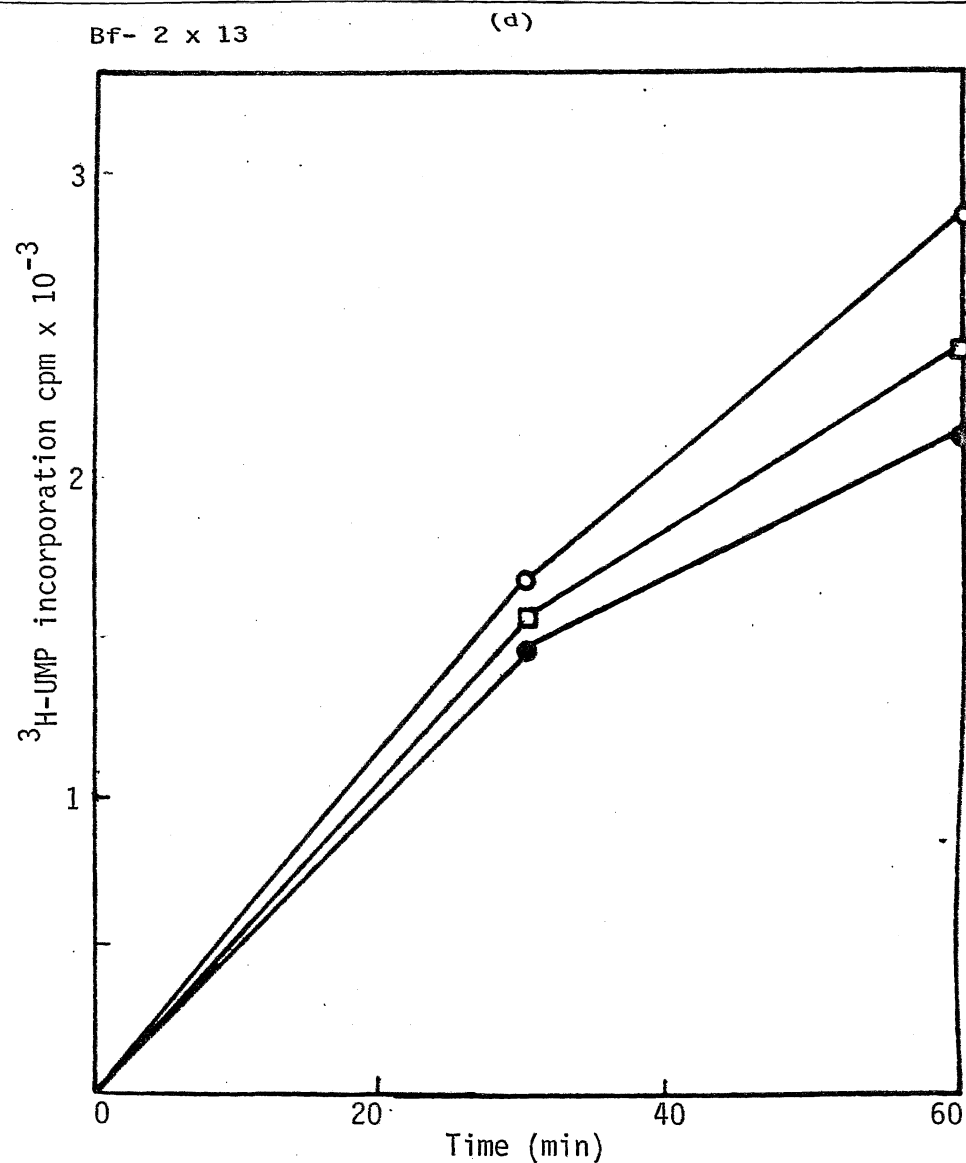
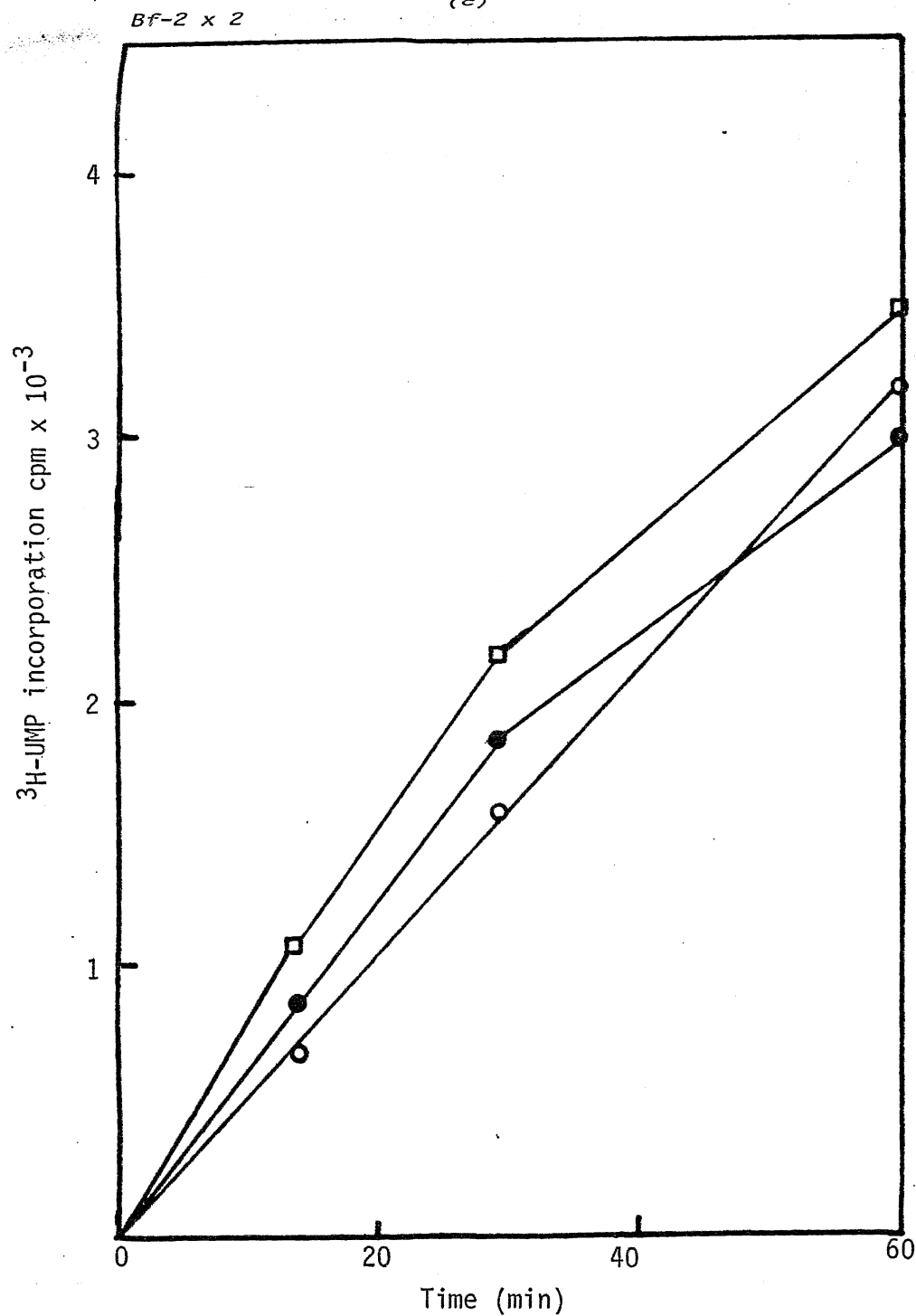


Fig 6.2 (c) & (d) Effect of the Bf-1 bacteriocin and rifampicin on RNA polymerase activity in Rif^R mutants (a) Bf-2 x 2 and (b) Bf-2 x 13. Legends are as in Fig 6.2 (a) & (b).

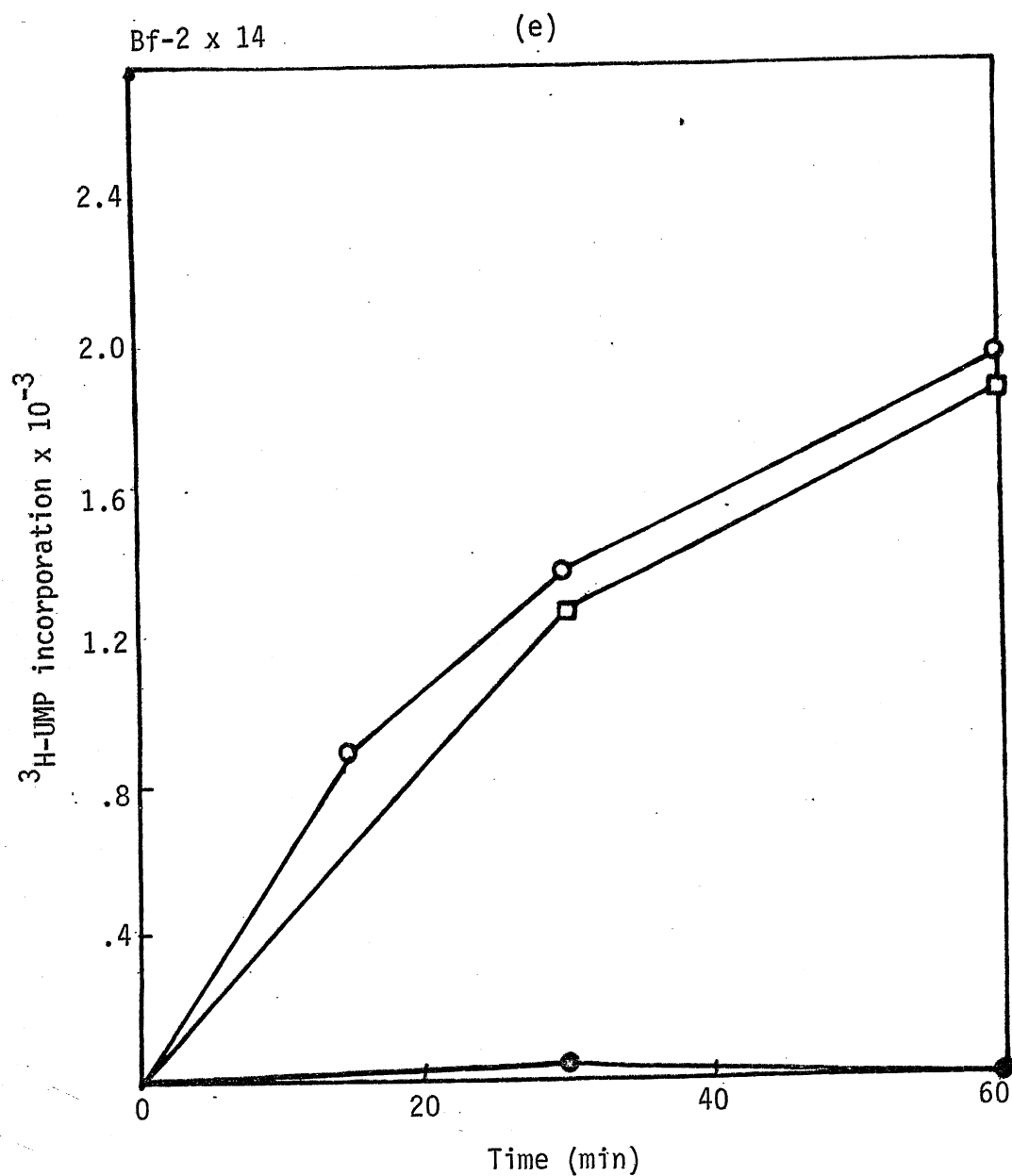


Fig. 6.2 Effect of the Bf-1 bacteriocin and rif on RNA polymerase activity in Rif^R mutant Bf-2 x 14. Legend as in Fig 6.2(a).

6.4 DISCUSSION

Studies on RNA synthesis strongly indicate that the inhibition of RNA synthesis of the sensitive indicator strain Bf-2 by the Bf-1 bacteriocin occurs both in vivo and in vitro. Moreover, the fact that RNA polymerase activity was repressed in sensitive strains upon the addition of the Bf-1 bacteriocin suggests that some aspect of RNA metabolism associated with this enzyme, or even the RNA polymerase itself, is the target of Bf-1 bacteriocin action. Since rif^R strains were found to be resistant to the action of the Bf-1 bacteriocin it is suggested that the mechanism of insensitivity to rifampicin could well be similar to that of tolerance to the Bf-1 bacteriocin. Since altered RNA polymerases have been implicated in the case of rifampicin resistance (Losick & Sonenshein, 1969; Sonenshein & Losick, 1970), it is plausible to suggest that the target of Bf-1 bacteriocin action is in fact the RNA polymerase itself. Furthermore, alteration in the RNA polymerase could well render the cell insensitive to bacteriocin Bf-1 action.

Whether the Bf-1 bacteriocin acts by binding to the RNA polymerase as rif does is not clear at this stage. However, a strong possibility exists that there may be overlapping mutation sites which may be responsible for joint rif and Bf-1 bacteriocin resistance on the sensitive strain. Mutant strain Bf-2 x 14 although rif^R is supersensitive to Bf-1 bacteriocin. This is probably a result of an altered RNA polymerase, which instead of being unable to bind to the Bf-1 molecule as is the case with rif, is altered in a way which facilitates Bf-1 bacteriocin binding, and amplifies the observed effects. The results with this mutant suggest that the bacteriocin and rif bind at different sites. However, more studies on the structure of these enzymes is necessary before any conclusions can be made

and work is currently underway in our laboratory to purify and study the subunit assembly of RNA polymerases from these mutant strains.

The bacteriocinogenic strain, Bf-1, is insensitive to its own bacteriocin. This is not uncommon among bacteriocin producing strains and is believed to be due to the presence in the cell of an "immunity substance" (Nomura, 1967; Bowman et al., 1971). Addition of higher bacteriocin multiplicities usually overcomes such immunity (Reeves, 1972) as we have demonstrated in vitro. It is unlikely that an altered RNA polymerase is involved since the Bf-1 strain is sensitive to rifampicin. The inhibition of RNA synthesis in the bacteriocin producing Bf-1 strain by high multiplicities of the bacteriocin is taken as further evidence for the mode of action of the Bf-1 bacteriocin.

CHAPTER VII

DETECTION OF PLASMID(S) HARBORED BY Bf-1 STRAIN

The bacteriocin producing strain, Bacteroides fragilis strain Bf-1 harbours a cryptic plasmid. This plasmid has been purified on caesium chloride-ethidium bromide equilibrium centrifugation and on a 5-25% sucrose density gradient and was found to co-sediment closely with the Col E1 plasmid. Attempts to cure Bf-1 of this plasmid were unsuccessful.

7.1 INTRODUCTION

Most bacteriocins studied to date appear to be coded for by extra-chromosomal elements called plasmids. Pneumocin genetic determinants, however, may be an exception. In one apparently unconfirmed study, they were shown to be chromosomally located (Mindich, 1966).

The first analysis of the genetic basis of bacteriocin synthesis was carried out by Fredericq & Betz-Barreu (1953 a & b). The observed that in Escherichia coli F^+ x F^- crosses with Col E1-K30 in either the donor or the recipient, synthesis of colicin E1-K30 behaved as an autonomous marker, unlinked to the chromosome (Fredericq & Betz-Barreu, 1953a, 1953b). This suggestion that certain colicins were determined by extrachromosomal elements was first confirmed by nongenetic methods for Col E1-K30 when the DNA was analysed in the electron microscope by Roth & Helinski (1967). After purification of the cell lysates, bacteriocinogenic factors, like other plasmids, are found to be largely in the form of supercoiled, closed-circular molecules (Helinski & Clewell, 1971). The fact that covalently closed circular molecules are thermodynamically limited in the amount of intercalative dyes, such as ethidium bromide (Radloff, Bayer & Vinograd, 1967), which they can bind has been used in the isolation of plasmid DNA.

Colicinogenic factor DNA molecules have been recently divided into two major groups (Hardy, 1975):

Group I Col factors, e.g. Col factors D-CA23, E1-K30 and E3-CA38 have molecular weights of approximately 5×10^6 daltons, enough DNA to code for about 10 proteins, each with a molecular weight of approximately 30 000 daltons.

Group II plasmids, e.g. Col factors B-K77, Ib-P9 and V-K30 have molecular weights between 62×10^6 and 94×10^6 daltons. This is enough DNA to code for 100 different proteins of molecular weights around 30 000 daltons. Most of the larger plasmids are transferable by conjugation (Hardy, 1975; Reeves, 1972).

Several methods have been used to indicate the plasmid location of a genetic marker. The irreversible loss of the phenotypic character has been taken as an indication of plasmid involvement although other explanations such as point mutations and phase variation could be involved. As a result, "curing" a bacteriocinogenic strain of the bacteriocinogenic character has often been used to indicate the plasmid location of that factor. A number of "curing" techniques have been used. Various treatments, including elevated temperatures (May, Houghton & Perret, 1964), thymine starvation (Clowes, Moody & Pritchard, 1965), exposure to mutagens (Willets, 1967) and dyes (Dajani & Taube, 1974; Gangliano & Hinsdill, 1970; Hirota, 1960; Hetten & Vogels, 1973; Warren et al., 1974). Failure to cure strains of the bacteriocinogenic character using, for example, acridine orange, acriflavine, rifampin, ethidium bromide, sodium dodecyl sulfate or growth at 42°C has sometimes been taken to indicate that the bacteriocin is not coded for by the plasmid. An example is the homofermentative Lactobacillus strain producing lactocin 27 (Upreti & Hinsdill, 1975). However, this is not always the case and strains

harbouring the Col I-P9 and Col E2-P9 factors could not be cured of their plasmids by treatment with acridine orange (Ozeki, Stocker & Smith, 1962). Failure to cure a bacteriocin producing strain does not necessarily imply that a plasmid is not involved.

Attempts were made to "cure" the bacteriocinogenic strain, Bacteroides fragilis (Bf-1) of its plasmid by treatments with acriflavine, acridine orange, ethidium bromide, SDS or growth at 42°C. These attempts proved unsuccessful. As a result, the plasmid isolated is still regarded as cryptic until satisfactory evidence to indicate its phenotype is obtained.

7.2 METHODS

7.2.1 Media and Chemicals as in the Appendix.

7.2.2 Radioactive labelling of Bacteroides

The method of Tinnell & Macrina (1976) was used to label the DNA of the Bacteroides strains. An overnight BHI-broth culture of the producer strain was pelleted, washed in glucose minimal media, pH 7.7 (Varel & Bryant, 1974), and using sterile syringes to ensure anaerobiosis, injected into 3 x 10 ml glucose minimal media in Hungate tubes to give a starting optical density at 600 nm ca. 0.1. The cultures were incubated in a waterbath at 37°C for 30 min before injecting $\{^3\text{H}\}$ -thymidine to give a final concentration of 10 $\mu\text{Ci ml}^{-1}$. Incubation was continued for 6 h before the cells were harvested and the DNA extracted.

7.2.3 Preparation of cleared lysates

The method of Guerry et al. (1973) for plasmid DNA isolation was used. The $\{^3\text{H}\}$ -labelled cells were pelleted, washed twice in TES buffer

(0.05 M NaCl, 0.05 M Tris and 0.005 M EDTA, pH 8) and then resuspended in 0.9 ml of TES buffer containing 1 mg lysozyme ml^{-1} and 10% sucrose. This suspension was then incubated at 37°C for 35 min to allow for the formation of osmotically fragile forms which were sensitive to lysis (Tinney & Macrina, 1976). The suspension was then kept at 0°C (swirling occasionally) for another 5 min after which lysis was brought about by the addition of 0.9 ml of lysis buffer (2% SDS in 0.01 M Tris pH 9). After 5 min the samples became relatively clear and viscous and 5 M NaCl was added to the viscous solution with gentle mixing to give a final concentration of 1 M NaCl. The lysates were stored at 4°C overnight after which they were centrifuged at 0°C for 30 min at 34 000 g, a step which normally pellets about 95% of the [^3H]-labelled chromosomal DNA while the plasmid DNA is recovered in the supernatant. This supernatant is hereto referred to as the "cleared lysate".

7.2.4 Equilibrium Centrifugation

Centrifugation was performed on a Beckman L2-65B ultracentrifuge in a type T1-65 fixed angle rotor at 105 000 g for 48 hours at 20°C. Each polyallomer tube contained 5.22 g CsCl, 1.6 ml of ethidium bromide (2 mg ml^{-1} in phosphate buffer pH 7), 3.1 ml sterile distilled H_2O and 0.8 ml sample ("cleared lysate"). The tube was then filled with light mineral oil. At the end of the run, 4 drop fractions were collected in a microtitre plate using a Buchler Polystatic Pump. Samples (25 μl) of each fraction were spotted on Whatman glass fiber paper (GFA) discs. The discs were washed twice in cold 5% TCA and once in 95% ethanol before being dried at 50°C for 2 h. The discs were then placed in glass vials containing 10 ml of scintillation fluid (PP0/2,5-Diphenyl-1,3-oxazole-toluene mixture) before being counted in a Beckman LS 3150 Liquid Scintillation Counter.

7.2.5 Sedimentation velocity centrifugation

Neutral sucrose density gradients were prepared as follows:

5 - 25% sucrose gradients containing 1.0 M NaCl, 0.001 M EDTA, 0.001 M Tris-HCl, pH 7.3 and 0.01 M β -mercaptoethanol were prepared. A 0.8 ml DNA sample was layered onto the gradient and centrifuged in a Beckman SW 50.1 rotor at 150 000 g for 2 h at 20°C. The gradients were fractionated and counted as described above.

7.2.6 Preparation of Marker DNA

Escherichia coli K12 strain JC411 harboring the Col E1 plasmid was utilized in the present study. This strain was grown in glucose minimal medium (Miller, 1972) containing 250 g/ml deoxyadenosine and 2.5 μ Ci $\{^{14}C\}$ -thymidine mL^{-1} . Cells were pelleted, washed and the DNA extracted as described above. The "cleared lysate" was purified by CsCl-ethidium bromide equilibrium centrifugation. The fractions representing the covalently closed circular (CCC) DNA were pooled, dialysed against TES buffer before being purified by centrifugation in a 5 - 25% sucrose density gradient as described above.

7.2.7 Elimination of plasmid DNA

An overnight BHI-broth culture of the producer strain was inoculated into BHI-broth containing ethidium bromide ($15 \mu g mL^{-1}$) acriflavine and acridine orange ($25 \mu g mL^{-1}$) SDS (0.04%) and incubated at 37°C for 8 h. The cultures were plated and the plates incubated at 37°C for 48 h. The colonies were replica plated onto indicator lawns of the sensitive strain to differentiate bacteriocin minus strains, as described in Chapter II.

7.3 RESULTS

7.3.1 Equilibrium centrifugation analysis of strain Bf-1.

Equilibrium centrifugation of the "cleared lysate" in caesium-chloride-ethidium bromide resulted in a radioactivity profile shown in Fig 1. A dense satellite peak (fraction 34) was observed. This peak represented the covalently closed circular (CCC) DNA and the other peak (fraction 46) represented both chromosomal and open circular (OC) DNA. The fractions representing the CCC DNA were pooled, dialysed against TES buffer to remove CsCl and ethidium bromide before being cosedimented with purified Col E1 DNA on a 5 - 25% sucrose density gradient. Figure 2 shows the pattern obtained. The peak at fraction 28 was assumed to represent the CCC DNA whilst the peak at fraction 34 was taken to represent the open circular (OC) DNA. The arrow indicates the position (fraction 35) of the Col E1 marker.

7.3.2 Elimination of bacteriocinogenic factor

Growth of bacteriocinogenic Bf-1 strain in the presence of curing agents did not eliminate the bacteriocin producing ability of this strain.

7.4 DISCUSSION

Clowes (1972) defined a bacterial plasmid as a genetic element which is physically separate from the chromosome of the cell and is able to be perpetuated stably in this condition. It is non-essential for growth of the normal cell so that under most conditions, it may be gained or lost without lethal effect.

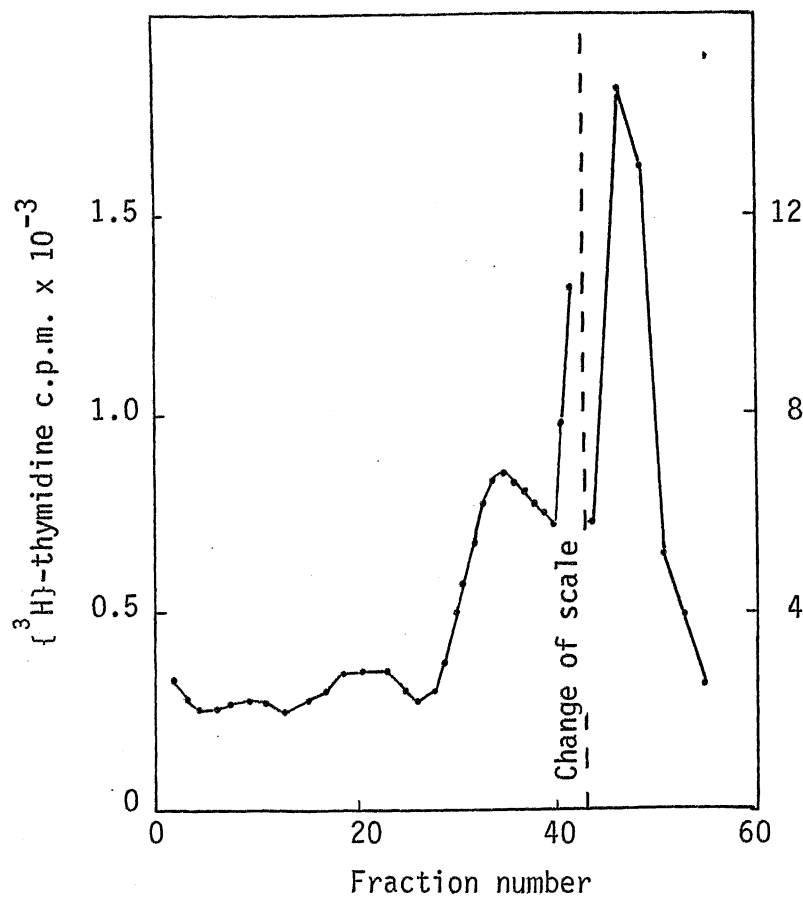


Fig. 7.1

^3H profile of CsCl-Ethidium bromide gradient of Bf-1. ^3H -thymidine-labelled lysates were precipitated and their supernatants subjected to CsCl-EB centrifugation as described in methods. Note the change of scale at fraction 43.

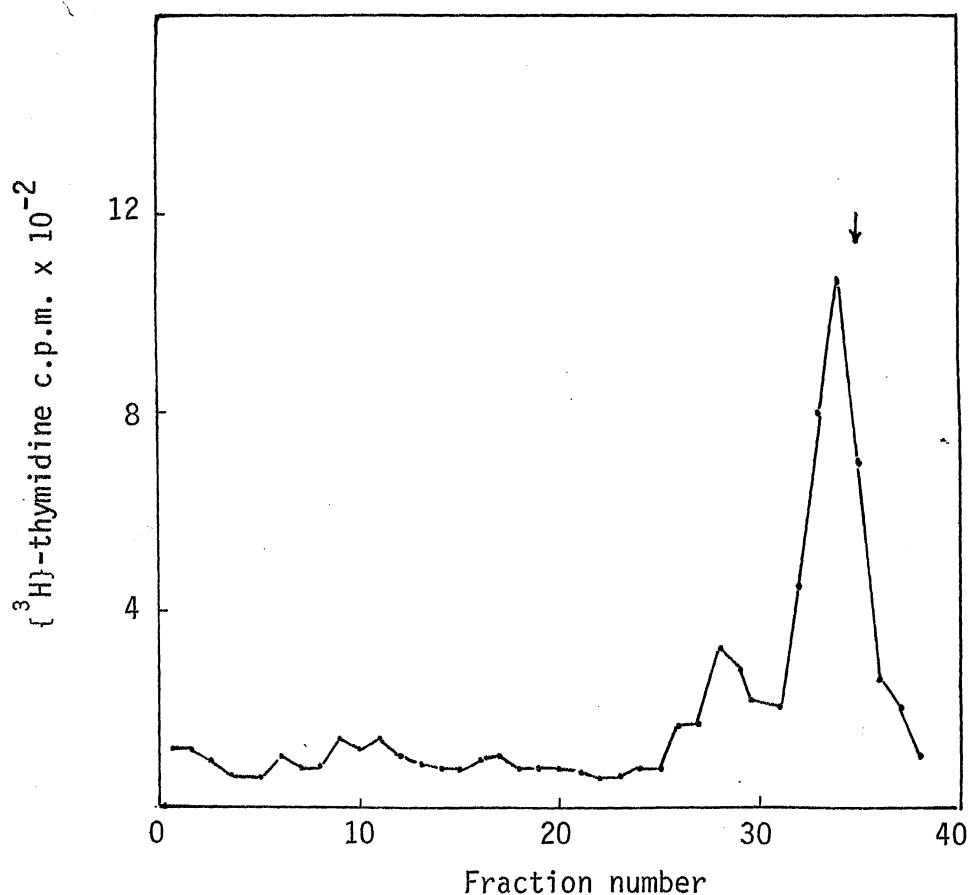


Fig. 7.2 Sedimentation of the Bf-1 plasmid DNA through a 5 - 20% neutral sucrose gradient. The arrow indicates the position where the Col E1 marker plasmid co-sedimented.

Bacteroides fragilis strain Bf-1 harbours a cryptic plasmid which sediments faster than the Col E1 plasmid DNA marker on a neutral 5 - 25% sucrose density gradient. This suggests that this cryptic plasmid is slightly larger than the 24S (molecular weight ca. 4.2×10^6 dalton) Col E1 plasmid. Since the purpose of this study was mainly to demonstrate the presence of plasmid DNA in bacteriocinogenic strain Bf-1, no attempt was made to characterize the plasmid further. The presence of plasmids in Bacteroides species has been demonstrated (Stiffler, Keller & Traub, 1974; Guiney & Davis, 1975; Tinnell & Macrina, 1976). In all instances, the small molecular weight plasmids were present in multiple copies per chromosomal equivalent, while the larger plasmids were present only in a few copies per chromosome. All these plasmids were regarded as cryptic and it is only recently that Guiney & Davis (1978) identified a conjugative R plasmid in Bacteroides ochraceus. Attempts to "cure" the bacteriocinogenic Bf-1 strain were unsuccessful. This does not necessarily rule out the plasmid nature of this phenotype and attempts are currently underway in our laboratory to confirm the phenotype of this plasmid by other means.

CONCLUSION .

The aim of this study was to isolate, characterize and to determine the mode of action of a bacteriocin produced by a Bacteroides fragilis strain. Although bacteriocins of Bacteroides strains have been characterized this is the first report on the mode of action of a Bacteroides bacteriocin. Bacteriocins produced by Bacteroides strains are interesting in that they appear to be somewhat unusual and unlike other well characterized bacteriocins. While Booth et al. (1977) reported on a large molecular weight (> 300 000 daltons) bacteriocin which was unusually resistant to high temperature exposure, we (Mossie et al. 1979 - Antimicrobial Agents Chemother. in press) observed an unusual biphasic temperature stability in the Bf-1 bacteriocin studied (MW between 14 000 and 19 000 daltons). Whether this is a common feature for all Bacteroides bacteriocins is not clear at this stage.

The unusual mode of action of the Bf-1 bacteriocin could well suggest that Bacteroides bacteriocins form a unique class of their own and may not necessarily be grouped under any of the classes of other well studied bacteriocins. However, more studies on the mode of action of other Bacteroides bacteriocins is needed before any such conclusion in this regard can be made.

The insensitivity of rif^R mutants of sensitive Bf-2 indicator strain to the Bf-1 bacteriocin indicates the involvement of the RNA polymerase in bacteriocin action. This is the first report of a bacteriocin specifically inhibiting RNA synthesis in general, and affecting RNA polymerase activity in particular. Again, whether the Bf-1 bacteriocin binds to the RNA polymerase as is the case with rifampicin, will only be resolved when the RNA polymerases of these strains are fully characterized.

Besides being a potential tool for studying transcription in Bacteroides strains, the Bf-1 bacteriocin and rif^R mutants of the Bf-2 strain may be useful in devising a rapid screening method for determining the mode of action of other bacteriocins within this group.

APPENDIX

BACTERIAL STRAINS

The bacterial strains used are listed in Chapter II.

GENERAL METHODS

Organisms were grown at 37°C unless otherwise stated. Cells were pelleted in a Sorvall RC-5 centrifuge at 8 000 g for 20 min at 4°C.

Preparative ultracentrifugation was done in a Beckman Model L2-65B ultracentrifuge.

Radioactivity and light emission was measured in a Beckman LS 3150T Scintillation Counter.

A Pleuger CVC-E voltage and current regulated DC power supply was used for electrophoresis.

MEDIA

(a) Preparation of media

Pre-reduced and anaerobically sterilised (PRAS) media were prepared according to the principles of Hungate (1969) and Moore (1966).

The aim of PRAS media preparation is to drive off the oxygen and partially reduce the media with cysteine hydrochloride or sodium thioglycollate. The medium is then maintained in an anaerobic state by means of tightly-fitting rubber stoppers or by keeping it in anaerobic jars. Sterilization by autoclaving was at 15 lbs in⁻² for 15 min for conventional media and for 20 min for media in

Hungate tubes. Distilled water was used in all media. All percentage compositions were w/v unless otherwise stated.

(b) Media

Antibiotic gradient plates: Rifampicin ($20 \mu\text{g ml}^{-1}$ final concentration) was added to BHI-agar at 50°C . Aliquots (10 ml) containing rifampicin were poured into petri dishes and allowed to solidify in a slanted position. BHI-agar (fresh) (10 ml) was then poured onto this to give a flat surface.

Note: Rifampicin is dissolved in 95% ethanol (1 ml per mg rif).

Brain heart infusion agar (BHIA)

Brain heart infusion broth	3.7 g
Yeast extract	0.5 g
Agar	1.5 g
H ₂ O	100 ml

Autoclave, cool to 50°C and add 1 ml cysteine hydrochloride, 2 ml sodium carbonate and 1 ml haemin-menadione stock solutions in that order.

Sloppy agar

Sloppy agar was made up as in BHIA plates, but with 0.7% agar. All ingredients were added together and boiled, perfused and autoclaved as for BHIB. It was freshly boiled before use.

Brain Heart infusion broth (BHIB)

Brain heart infusion broth	3.7 g
Yeast extract	0.5 g
Sodium thioglycollate	0.11 g
Sodium carbonate	0.4 g
Resazurin stock solution	1.0 ml
H ₂ O	100 ml

The broth mixture was heated until colourless, dispensed into Hungate tubes and 0.1 ml hemin-menadione stock solution added per 10 ml broth. It was then bubbled with CO₂ + H₂ according to the method of Kistner (1960). The tubes were autoclaved for 20 min.

Cysteine hydrochloride stock solution

Cysteine hydrochloride (0.5 g) was dissolved in 10 ml water and autoclaved. This was added to anaerobic media (BHIA) to give a final concentration of 0.05%.

Hemin-menadione stock solution (H-M)

Menadione stock solution: Add 100 mg menadione to 20 ml 95% ethanol. Filter-sterilize and store at 4°C.

Hemin stock solution: Add 50 mg hemin to 1 ml 1N NaOH. Make up to 100 ml with distilled water. Autoclave.

Minimal Medium (MM)

<u>Mineral solution:</u>	KH_2PO_4	18 g
	NaCl	18 g
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.53 g
	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.4 g
	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.2 g
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.02 g
	H_2O	

Dissolve the salts in the order given in distilled water.

Autoclave and store over chloroform at 4°C .

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: Stock solution 4 g/100 ml. Add to media to give final concentration of 0.0004 g/ml. Store over 10% chloroform at 4°C .

Ammonium sulphate stock (6 mM): Dissolve 0.79 g in 1000 ml water and autoclave.

Glucose/lactose stock: Final concentration 0.5 g per 100 ml media. Stock 10 g/100 ml. Autoclave, and add 5 ml to media.

Vitamin B_{12} stock solution: Stock solution 10 mg ml^{-1} .

Add 0.5 ml to media to give final concentration of 0.05 mg/100 ml.

Minimal medium broth

Mineral solution	5 ml
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1 ml
$(\text{NH}_4)_2\text{SO}_4$ (6 mM)	5 ml
Resazurin indicator	1 ml
H_2O	79 ml

Autoclave, and add:

Sterile glucose/lactose	5 ml
" vitamin B ₁₂	0.5 ml
" hemin-menadione	1 ml
" cysteine-hydrochloride	1 ml
" sodium carbonate stock or	
" 0.1 M phosphate buffer	2 ml

Dispense into sterile tubes in 10 ml amounts and gas.

Minimal media supplemented with BHIB

This media was used for uptake experiments. 1 ml of BHIB is added to 2 ml of glucose minimal media.

Resazurin stock solution: Dissolve 20 mg resazurin in 10 ml distilled water. Add 1 ml of stock solution to 100 ml medium

Semi-solid indicator:

48 drops 9% sodium thioglycollate
 20 ml 2% boraz
 75 ml distilled water

0.7 g agar

0.2 mg resazurin

The mixture is boiled until colourless, dispensed into Astell roll tubes and perfused with $\text{CO}_2 + \text{H}_2$ mixture.

Sodium carbonate (Na_2CO_3) stock solution: Na_2CO_3 (Hg) is dissolved in 20 ml distilled water and autoclaved. This is added to anaerobic media to give a final concentration of 0.4%.

Standard saline solution: Sodium chloride (0.85 g) was dissolved in 100 ml distilled water and autoclaved.

Scintillation cocktail:

10 x solution: Primary fluor diphenyl oxazole PPO	16.7 g
Toluene	1000 ml

Store in the dark. Dilute 10 x with toluene before use.

CHEMICALS AND REAGENTS

Antibiotics:

Chloramphenicol	Sigma, St. Louis, Mo., U.S.A.
Rifampicin	

Chromatographic chemicals:

Blue dextran 2000	Pharmacia, Uppsala, Sweden
Sephadex G-100	
DEAE-cellulose DE-52	Whatman, U.K.

Dyes, stains and indicators:

Acridine orange	BDH Chemicals, Poole, U.K.
Acriflavine	
Ethidium bromide	
Resazurin	
Coomasie Blue	Merck, Darmstad, Germany

Enzymes:

Deoxyribonuclease	Sigma, St. Louis, Mo., U.S.A.
Luciferin-luciferin-luciferase FLE-50	
Pronase	Miles Seravac, Cape Town, S.A.
Ribonuclease	
Trypsin	
Lysozyme	

Media:

Brain heart infusion broth	Difco, Detroit, Michigan, U.S.A.
Yeast extract	
Agar	

Radiochemicals:

{8- ³ H} adenine (25 Ci mmol ⁻¹)	Amersham Radiochemical Centre,
L-{U- ¹⁴ C} glycine (112 mCi mmol ⁻¹)	Amersham U.K.
L-{4,5- ¹⁴ C} leucine (146 Ci mmol ⁻¹)	
{U- ¹⁴ C} protein hydrolysate (57 mCi mAtom ⁻¹)	
{6- ³ H} thymidine (22 Ci mmol ⁻¹)	
{5- ³ H} uracil (44.5 Ci mmol ⁻¹)	
{5- ³ H} UTP (23 Ci mmol ⁻¹)	

Vitamins:

Insulin	Sigma, St. Louis, Mo., U.S.A.
Hemin chloride	Fluka AG, Bucks SG, Switzerland.
Menadione-sodium bisulphite	Merck, Darmstad, Germany
Vitamin B ₁₂	

General chemicals:

Adenosine-5'-triphosphate	Sigma, St. Louis, Mo., U.S.A.
Phenylmethylsulfonyl fluoride	
Ovalbumin	
Caesium chloride	Merck, Darmstad, Germany
Ethylenediaminetetraacetic acid	
Dithiothreitol	
Glycerol	
2-Mercaptoethanol	
Toluene	
2.5-Diphenyloxazole PPO	Beckman, U.S.A.
2.2'-p-phenylbis (5-phenyl oxazole)POPOP	
Cyanogum 41	BDH Chemicals, Poole, U.K.
Sucrose	
Sodium arsenate	
Sodium thioglycollate	
3-Dimethylaminopropionitrile	BDH Chemicals, Poole, U.K.
Bovine Serum albumin	Koch-Light, Colubrook Bucks, U.K.
Sodium dodecyl sulfate	Hopkin & Williams, Essex, U.K.
Ammonium persulfate	M & B, Dagenham, U.K.

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