TITLE PAGE

MOLECULAR GENETIC STUDIES

OF

BACTEROIDES FRAGILIS

BY

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SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE

DEGREE OF

Ph.D

IN THE FACULTY OF SCIENCE

UNIVERSITY OF CAPE TOWN

CAPE TOWN

MARCH 1986

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In terms of the paragraph of "General regulations for the degree of Ph.D" I, as supervisor of the candidate J.A.Southern, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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MOLECULAR GENETIC STUDIES OF BACTEROIDES FRAGILIS

CONTENTS

SUMMARY

Some genetic systems operative in Bacteroides fragilis have been successfully investigated.

Firstly the bacteriocin produced by the B.fragilis BF-1 strain was purified and partially characterized. This bacteriocin was found to be cell bound and constitutively produced by the bacteria. The purified bacteriocin was a protein with an apparent Mr of 6400-7200, and was relatively heat stable. The action of the bacteriocin resulted in the lysis of sensitive bacteria.

As previous reports indicated that a bacteriocin isolated from the BF-1 strain inhibited the action of RNA-polymerase in-vivo and in-vitro, RNA-polymerase was extracted and partially purified from the bacteriocin sensitive B.fragilis BF-2 strain (described in Appendix 5). This enzyme was essentially similar to that described for other Eubacteriales, but was not inhibited in the in vitro assay by the purified bacteriocin described in this thesis.

A cryptic plasmid was discovered in a B.fragilis strain and was extracted, purified and a partial restriction map established. The plasmid had an approximate size of 6.0 kilobases.

By genetic manipulation, hybrids of this plasmid and the vector, pBR325 from Escherichia coli, and the clindamycin resistance determinant from another strain of B.fragilis were constructed with the aim of developing a plasmid which could replicate in both species of host bacterium. It was not possible to transform this hybrid plasmid, or other Bacteroides plasmids into the available strains of B.fragilis.

A gene bank of B.fragilis chromosomal DNA was established in the E.coli vector plasmid pEcoR251, and this library was screened by complementation of known E.coli mutant and deletion strains. Plasmid clones were isolated which complemented auxotrophic markers and another which appeared to confer resistance to mitomycin C.

A plasmid clone (pJS139) was isolated which complemented the glutamine synthetase deficiency of an E.coli deletion strain. The DNA insertion proved to be derived from B.fragilis, and was 8.7 kilobases in size. The position of the glnA gene was determined to be on a 4.7 kb segment of the DNA insertion. The regulation of expression of the glutamine synthetase produced by the E.coli clone containing pJS139 was studied. This showed that the production of active enzyme was regulated by the presence of available nitrogen in the growth medium, but that this regulation differed from the E.coli wild type enzyme.

The cloned gene was not able to co-regulate the E.coli high affinity arginine and glutamine uptake pathways, nor could it activate the hut operon of Klebsiella aerogenes carried by this E.coli strain.

Finally the glutamine synthetase enzyme produced by this recombinant plasmid was extracted and purified, and the characteristics of the enzyme established. It proved to have a subunit Mr of approximately 75000 and a holoenzyme Mr of 490000. Electron microscopy showed molecules with the ring shaped structure typical of other glutamine sythetase enzymes which have been described. In contrast to most other Eubacterial glutamine synthetase enzymes which have been described, which consist of twelve sub units, this cloned enzyme appears to consist of only six sub units.

There was no evidence for the adenylylation/deadenylylation control of this enzyme which has been described for aerobic Gram-negative organisms, but there was evidence for a requirement for some form of phosphorylation of the molecule for activity.

Crude extracts of B.fragilis were found to contain small amounts of glutamine synthetase activity and a potent inactivator of this enzyme function.

ACKNOWLEDGEMENTS

I wish to acknowledge the support of the Department of Health and Welfare, which has made this project possible within the framework of its Research and Development programme at the State Vaccine Institute.

I wish to thank Professor D.R. Woods for his enthusiasm, and encouragement while supervising this project. My thanks to the academic and technical staff of the department of Microbiology of the University of Cape Town for technical assistance and helpful discussions. In particular Harold Zappe, Dr Petra Schumann, Dr Heide Goodman, Prof. Joan Parker, Dr Doug Rawlings, Dr Karen Usdin, Mary-Lou Hunt, Ernest Clark, Eric O'Neil and many others that have helped in one way or another.

My thanks to the staff of the Vaccine Institute in particular the Director Dr Wolf Katz who encouraged me to pursue this project and who advised throughout the work and while preparing this thesis. My thanks to Mrs Delia Smith for excellent technical assistance, and to Emlyn Hitchings for help with the photography and illustrations, and to Barbara Wray and Dr Linda Stannard for help with the electron microscopy.

To my wife, Sue, and sons, James and Richard, who have had to live with me.

CHAPTER 1.

AN INTRODUCTION TO THE MOLECULAR GENETICS OF BACTEROIDES FRAGILIS

The genus Bacteroides is defined as Gram-negative, obligate anaerobic, non-sporing, sometimes encapsulated, usually non-motile rods. They have a DNA base composition of 41-43 mol% (G+C). The members of this genus are colonists of man and animals. Animal bodies have many suitable environments for the growth of these organisms which are found in significant numbers and are often the major proportion of the microbial population (Moore et al., 1969; Edmiston et al., 1982).

The effects which these organisms may have on their host are many, ranging from digestion of cellulose in ruminants (Wallace & Bramall, 1984), through the in vivo production of anti-microbial metabolites (Morin et al., 1982), to the causation of periodontal disease and gigivitis (Yoshimura & Nishikata, 1984; Moore et al., 1985).

The present taxonomy of the Bacteroides fragilis group of organisms was established by Holdeman & Moore (1972) and Cato & Johnson (1976). On the basis of DNA homology they were able to define the members of what had previously been known as the B.fragilis group, as distinct species. This group includes B.fragilis, Bacteroides thetaiotaomicron, Bacteroides ovatus, Bacteroides distasonis, Bacteroides uniformis and Bacteroides vulgatus.

Antigenic analysis of this group has shown no distinct species antigen but rather a mosaic of different antigens on each strain, which may cross react with each other (Babb & Cummins, 1981; Tabaqchali et al., 1982).

B.fragilis is the major pathogen of this group. The encapsulated strains are opportunist pathogens, and are the most frequent isolates from pathological conditions (Kasper et al., 1979), although they have been found to constitute less than 0.5% of the colonic microflora. They are usually pathogenic as members of a mixed infection where the rapid metabolism of other opportunistic aerobic and facultatively anaerobic micro-organisms generate the anaerobic conditions suitable for growth (Dunn & Barke, 1985).

The significance of the capsule and capsular antigens to the virulence of B.fragilis strains has been investigated by Kasper et al. (1979) who found that the presence of certain surface (probably capsular) antigens correlated with virulence. Connolly et al. (1984) consider that this is due to the inhibition of phagocytic bacterial killing by the capsular material.

However, Reid & Patrick (1984) have evidence that encapsulated strains are not more protected from phagocytic killing, but that these strains are more tolerant of exposure to air (Patrick et al., 1984).

The pili of B.fragilis appear to be concerned with the attachment of the bacterium to host cells and to polysaccharide substrates (Pruzzo et al., 1984; Salyers, 1984).

B.fragilis has been implicated in the aetiology of colon cancer. Van Tassel et al. (1982) found the it could reduce nitrate containing substrates to carcinogenic nitrite compounds. The production of potential carcinogens by reaction with bile salts in the large intestine has also been reported (Hill et al., 1971).

Bacteroides species, and B.fragilis in particular, inhabit body sites which are also colonized by many other bacterial groups, and the possible transfer of antibiotic resistance between these groups is of considerable medical importance. Strains of B.fragilis are frequently found which are resistant to many of the commonly used antibiotics (Sutter & Finegold, 1976; Cuchural et al., 1984), and the transfer of these genes to other species is suspected (Tally et al., 1979). The protection of penicillin sensitive pathogenic Streptococci by the presence of resistant B.fragilis in a mixed culture, has also been described (Brook & Yokum, 1983).

The molecular biology and genetic mechanisms of B.fragilis are of considerable medical interest and of more general interest as examples of a highly adapted anaerobic metabolism.

The choice of B.fragilis as a model for the study of genetic mechanisms of anaerobes has been outlined by van Tassel & Wilkins (1978). First, it has clinical importance, and second, it is less difficult to experiment with than other anaerobes, as it will tolerate prolonged exposure to air, and there are established complex and defined media for its cultivation (Varel & Bryant, 1974). Bacteriophages specific for B.fragilis have been isolated and studied as have a number of auxotrophic mutants.

The effects of oxygen have been studied in B.fragilis and the suggested mechanisms by which it protects itself from oxygen damage have been described (reviewed by Woods & Jones, 1986). Oxygen is toxic because of the potent oxidizing radicals formed during the reduction of molecular oxygen. These toxic intermediate products are peroxide (H_2O_2), the superoxide radical (O_2^-), and the hydroxyl radical (OH°). They may cause oxidative damage to intracellular components in particular DNA where single strand breakages have been noted.

Micro-organisms possess systems of enzymes which are capable of inactivating peroxide and the superoxide radical. Catalases and peroxidases dissociate peroxide to form molecular oxygen and water, while the superoxide dismutase (SOD) group of enzymes convert the superoxide radicals into peroxide and water.

Privalle & Gregory (1979) have shown that SOD enzymes are present in B.fragilis and that they are induced by exposure to oxygen. In B.fragilis peroxide and oxygen have been shown to stimulate DNA repair mechanisms (Slade et al., 1983a) and to induce the production of a number of specific polypeptides (Schumann et al., 1982). Thus these anaerobes are not without defences against the toxicity of oxygen.

Aerobic organisms take advantage of the oxidizing power of oxygen to generate energy. The respiration of a facultative anaerobe such as Escherichia coli is extremely complex (reviewed by Ingledew & Poole, 1984). There are three principal respiratory chains, based on fumarate, nitrate and oxygen, which yield -16, -39 and -52 Kcal/M respectively. Thus the ability to use oxygen gives the bacterium access to considerably more energy from the same quantity of substrate than it could acquire by either of the two anaerobic pathways.

GENETIC MECHANISMS IN BACTEROIDES SPECIES

The study of genetic mechanisms in anaerobic bacteria has lagged considerably behind that of the aerobic bacteria. This has been mainly due to the technical problems in maintaining the strict anaerobic conditions required for growth and to a general lack of knowledge concerning the biology of the organisms concerned.

The local development (Moodie & Woods, 1973) and commercial availability of anaerobic glove cabinets has allowed the "bench" manipulation of micro-organisms under the strict anaerobic conditions required for their viability. The most important innovation has been the incorporation of catalytic cartridges into the incoming gas lines to remove traces of oxygen from the compressed gasses used to maintain the anaerobic atmosphere in the cabinet.
The recent progress in the understanding of the genetics of these organisms are a result of these technical advances.

Genetic mechanisms which are analogous to those studied in aerobic organisms have been sought, and although there are several which have been reported, the interpretation of these must be taken as tentative if based solely on similar phenomena in the aerobes.

BACTERIOPHAGE, LYSOGENY AND TRANSDUCTION

The analysis of the processes involved in bacteriophage reproduction assisted in the understanding of molecular genetics in micro-organisms (Cairns et al., 1966). The presence of bacteriophage has been noted within the Bacteroides genus. Nadescu et al. (1972) isolated and studied two B.fragilis phages from sewage and studied their characteristics. They reported apparent induction of phage from mixed cultures of bacteria. This suggested the presence of lysogeny although they were unable to find a pure culture of B.fragilis from which phage could be induced. Brandis et al. (1972) studied the morphology and properties of one of these phages and found that it belonged to morphological group B of Bradley (1967) and resembled the coliphage λ in its biological properties.

A phage isolated from sewage by Burt & Woods (1977) was specific for B.thetaiotaomicron and also exhibited apparent lysogeny in this strain, however passage of the carrier strain through specific phage antiserum eliminated the carrier state. Phage could not be induced by Ultra-violet (UV) irradiation, mitomycin C, temperature or aging and it was concluded that true lysogeny had not been observed. Successful transfection of B.thetaiotaomicron with DNA extracted from this phage was reported. The apparent lysogeny (pseudolysogeny) which was observed was suggested to be due to the continual development of phage-sensitive bacteria from a phage-resistant population.

A similar phenomenon had been reported by Keller & Traub (1974) who studied a phage of B.fragilis.

Booth et al. (1977) reviewed the findings regarding Bacteroides phages. Of the 67 distinct phages which had been studied (of 4 morphological types of Bradley (1967)), they could find no definite evidence for lysogeny in this group.

This corresponded to the findings of Jones (1980), who found that all 12 of the B.fragilis phages which he had examined were virulent, and that none of the 36 B.fragilis strains which he had collected could be induced to produce phage and that the state of pseudolysogeny was associated with the production of capsular material which protected the cells from bacteriophage infection. Non-encapsulated variants were frequently segregating from the population and these proved to be susceptible to the phage.

No evidence for phage transduction of antibiotic or auxotrophic markers was detected in B.fragilis.

Silver et al. (1975) reported phage-like particles in thin sections of B.fragilis, but they did not detect lysis of the carrier strain in culture, nor could they find any other strain which was sensitive to these particles. Other intracellular structures in B.fragilis have been reported by Reid (1981). These may be temperate phage or some other, as yet unexplained, phenomenon particular to B.fragilis.

Lysogeny and transduction have been important mechanisms for the study of the genetics of E.coli and other micro-organisms, but they do not appear to be available for the study of B.fragilis.

HOMOLOGOUS AND ILLEGITIMATE RECOMBINATION

The mechanisms by which regions of homologous DNA are recombined have been extensively studied in E.coli and Bacillus subtilis (Stent & Calender, 1978). These mechanisms result in the processes of generalized transduction, and recombination following the transformation of competent bacteria with DNA. There have been no reports of similar phenomena in B.fragilis (Woods & Jones, 1986).

There is evidence in B.fragilis for the illegitimate recombination of transposable elements which can insert into several sites of a genome. Insertion sequences (IS) are defined as sequences of DNA which contain no known genes unrelated to their insertion function and are generally shorter than 2 kb. Transposons (Tn) are more complex elements, which often contain IS elements (usually at either end), and behave formally like IS elements but contain additional genes unrelated to insertion function; they are generally larger than 2 kb (Campbell et al., 1977).

The resistance tranfer factors pBF4 (Welch & Macrina, 1981) and pBFTM10 (Tally et al., 1982) were found to contain regions of DNA homology only within the the DNA fragment known to contain the clindamycin-erythromycin resistance determinant (Shimell et al., 1982; Guiney et al., 1984b). These resistance plasmids are very different in size and were isolated from clinical specimens in France and the United States of America, respectively.

Shimell et al. (1982) also found evidence that the clindamycin resistance gene (Cc^r) was flanked by direct DNA sequence repeats containing restriction enzyme sites for EcoRI and AvaI. Homology to this segment of plasmid DNA was also detected in chromosomal digests of DNA from clinical isolates of Bacteroides species which were Cln^r (Marsh et al., 1983)

Shoemaker et al. (1985) constructed a hybrid plasmid (pSS-2) which could replicate in E.coli. This contained a 33 kb fragment from pBF4, which includes the Cc^r region.When this plasmid was mobilized by R751 between E.coli strains, R751 DNA from these transconjugant strains was subsequently isolated and contained a 5.5 kb segment of DNA which showed homology to the Cc^r gene of pBF4.

This segment was found to be inserted into one of several sites in the co-resident plasmid and this same 5.5 kb fragment was also found to be inserted into the E.coli chromosome. Transfer was recA independent and was also noted to occur a second time from chromosomal to plasmid DNA.

The corresponding Cc^r gene of pBFTM10 has been studied in detail (Robillard et al., 1985) and the resistance determinant was found to be carried by a compound, 5.6 kb transposon (Tn4400) which had active IS elements as directly repeated sequences at its ends. The pBFTM10 fragment containing this Cc^r determinant was inserted into a plasmid capable of replication in E.coli. This hybrid plasmid could be mobilized using an F factor derivative (pOX38) between E.coli strains.

Analysis of the products of mobilization showed that Tn4400 mediated cointegrate formation between the hybrid plasmid and the conjugal mobilizer. This took place at low frequency (5.5×10^{-8}) and could involve the entire Tn or one of the IS elements at its ends. Transformation of the cointegrate formed between the hybrid resistance plasmid and the conjugal mobilizer, into $recA^{+}$ E.coli strains resulted in the isolation of the original hybrid resistance plasmid and a derivative of the pOX38 which contained one or the other of the IS elements derived from Tn4400.
Thus this B.fragilis transposon functions in E.coli.

Robillard et al. (1985) also report briefly that they have been able to mobilize a plasmid containing Tn4400 into B.fragilis using an RP4 derivative and that Cln^{r} transconjugants could be detected which did not contain the plasmid and that the formation of auxotrophs in Cln^{r} transconjugants occurred at a frequency of 0.5 to 1%, indicating that insertional events were causing the inactivation of B.fragilis genes.

MUTAGENESIS AND DNA REPAIR

The repair of DNA in aerobic organisms has been reviewed by Walker (1984) and Friedberg (1985), and several systems have been described.

Pre-replication repair: The damage to the DNA molecule is repaired without overall replication of the whole cell genome.

Photoreactivation in E.coli is mediated by the phr gene product which specifically binds to pyrimidine dimers, absorbtion of light energy (310-480nm) photolyses the complex and repairs the damaged thymine bases and no DNA synthesis is necessary. Photoreactivation has not been detected in B.fragilis (Jones, 1980).

Recombination repair requires a recombination event between two homologous segments of DNA. The recA system is directly involved in this form of repair and is activated by DNA damaging agents. No homologous recombination has been detected in B.fragilis.

Excision repair: specific excision of UV induced pyrimidine dimers and an area around the lesion by bacterial endonuclease enzymes followed by resynthesis of the gapped region using the intact complementary strand as a template results in a repaired DNA sequence. There are two reported pathways of repair in E.coli. The short-patch repair pathway (Youngs et al., 1974), is believed to be responsible for most excision repair. It is constitutive and error-free, while the long-patch (up to 1500 nucleotides and also mediated by recA and lexA) is less understood.

Error-prone repair: This occurs in E.coli and permits the bypass of pyrimidine dimers which have not been excised from the DNA before replication occurrs. Error-prone repair (also mediated by recA and lexA) permits bypass of dimers during replication at the expense of replication fidelity and is responsible for most UV- induced mutagenesis (Castelazzi et al., 1980).

In E.coli, the recA system is a complex interacting web of enzymes which are induced by a variety of agents and forces (Walker, 1984) which damage DNA, such as UV irradiation, heat shock and exposure to chemicals. These enzymes induce the error-prone repair of damaged DNA, the induction of colicin production, the excision of chromosomally integrated prophage and the recombination of homologous DNA fragments. All these activities have not been reported in B.fragilis.

Droffner & Yamamoto (1983) investigated the effect of stringent anaerobic conditions on mutagenesis in Salmonella typhimurium and found that error-prone repair did not occur under these conditions. The recA and recB functions were not being expressed and they proposed that this was caused by a change in the physiological condition of the cell, preventing expression of the error-prone repair systems. Yamamoto & Droffner (1985) isolated S.typhimurium mutants, some of which could only grow aerobically while others required strict anaerobic conditions for growth.

The aerobic mutants were deficient in DNA gyrase enzyme function while the anaerobic mutants lacked DNA-topoisomerase I activity. They suggest that these enzymes control the expression of other genes by altering the conformation of the cellular DNA, and the particular genes affected by these mutations are the SOD and catalase required for growth in an aerobic environment. The changes in gene expression are suggested to be due to the state of of the cellular DNA (relaxed or supercoiled) which is modified by the topoisomerase and gyrase enzymes.

DNA repair mechanisms do exist in B.fragilis. The model used by Slade et al. (1983a, 1983b, 1984) has been the bacterial host cell mediated reactivation (HCR) of UV irradiated phage particles which cannot replicate in the bacterial host without repair to their damaged DNA. Pretreatment of B.fragilis cells by sub-lethal exposure to UV, oxygen or hydrogen peroxide enhanced the subsequent production of viable phage from cells which were infected with suspensions of irradiated phage. The responses of the B.fragilis repair to these three agents differed, indicating the possibility that at least three different repair systems may operate in this organism. In E.coli UV induced reactivation of phage is known as Wiegle reactivation (Bernstein, 1981) and is part of the error-prone, recA regulated repair mechanism.

The effects of UV on the survival of B.fragilis cells suggest a complex repair system. Exposure to short-wave UV (far UV) was more lethal to B.fragilis under aerobic than anaerobic conditions (Jones et al., 1980). This contrasted with findings in E.coli where the effects of far UV are independent of the effects of oxygen (Webb, 1977).
Liquid holding recovery however, of irradiated B.fragilis cells occurred under aerobic conditions but not anaerobically (Jones & Woods, 1981). Oxygen enhanced survival of B.fragilis cells which had been treated with DNA damaging agents such as n-methyl-n'-nitroso guanidine, ethylmethane sulphonate, acriflavin or mitomycin C was reported by Slade et al. (1984).

Exposure of B.fragilis to near UV irradiation, under anaerobic conditions, results in repair which is similar to that of E.coli (Peters & Jagger, 1981). Irradiation of B.fragilis cells under aerobic conditions resulted in a "V" shaped survival curve, this did not resemble the survival of wild type E.coli but did resemble that of recA mutants (Slade et al., 1982).

These results are evidence of UV and oxygen induced DNA repair systems operative in B.fragilis. There was however no increased incidence of mutant phage or bacterial cells detected during these experiments. The repair of irradiated B.fragilis under aerobic conditions resembles that of recA mutants of E.coli.

In contrast to the effects of UV irradiation on E.coli wt, irradiation of B.fragilis with far UV resulted in immediate, rapid and extensive cellular DNA degradation which continued for 40-60 min after anaerobic irradiation (Schumann et al., 1984). During this time DNA synthesis continued at a low level compared to unirradiated cells. E.coli recA cells show this form of response to UV irradiation. Protein synthesis was required for the DNA degradation of B.fragilis suggesting the presence of a UV induced DNA repair system operative in these cells. Shumann et al. (1982) reported that after UV irradiation under aerobic conditions extensive DNA degradation was not observed, which suggests the inhibition of the putative induced repair system.

In spite of the reported difficulties in obtaining suitable B.fragilis mutants for analysis (Van Tassel & Wilkins, 1978), derivatives of B.fragilis BF-2 have been obtained following treatment with ethylmethane sulphonate. These have altered survival properties following treatment with DNA damaging agents (Abratt et al., 1985). One of these mutant strains had an increased sensitivity to mitomycin C while maintaining a wild type resistance to the effects of UV irradiation. Another was only moderately sensitive to mitomycin C but was more sensitive to UV irradiation. Both strains exhibited liquid holding recovery following UV irradiation but showed impaired host cell reactivation of irradiated phage.

The level of this host cell reactivation could be stimulated by treatment of the cells with UV or peroxide. These results indicate several interrelated pathways of recovery from DNA damage.

Analysis of the ability of these mitomycin C and UV mutants to excise thymine dimers from irradiated DNA (Abratt et al., 1986) under various conditions indicated that although the UV mutant had reduced levels of dimer excision, this was not the case for the mitomycin C mutant. These effects were not influenced by the presence of oxygen and they suggest that dimer excision is not the mechanism involved in aerobic liquid holding recovery of B.fragilis.

RESISTANCE PLASMIDS, CONJUGATION AND GENETIC TRANSFER

The nomenclature of plasmids is based on that proposed by Novick et al. (1976).

A plasmid is a replicon which is stably inherited (without specific selection) in an extrachromosomal state.

When the plasmid is able to exist either integrated with the host bacterial chromosome, or as an extrachromosomal element, it is known as an episome. Plasmids which have no detectable phenotypic trait are known as cryptic plasmids and these have been found in all bacteria where plasmids are known to exist.

Some plasmids can mediate the transfer of genetic information between bacteria. These conjugative plasmids may integrate into the bacterial chromosome and cause the transfer of bacterial genes during conjugation (fertility of F plasmids). Resistance plasmids (R factors) carry genetic information for resistance to antibiotics or other antibacterials. They may or may not be conjugative plasmids. The rapid evolution of these resistance transfer factors (reviewed by Foster, 1983) has great importance for medical microbiology, as the treatment of bacterial infections is frequently complicated by the emergence of pathogens which are simultanously resistant to several antibiotics.

Colicinogenic or bacteriocinogenic plasmids carry the genetic information for the production of a colicin or bacteriocin. They may also be conjugative plasmids (Hardy, 1975).

The map of extensive areas of the E.coli chromosome was established in a large measure using the biological activity of the fertility plasmid F1, a conjugative episome, described by Jacob & Wollman (1961).

Bacterial mating is mediated by conjugative plasmids carrying genetic information which directs the mobilization (mob) and transfer (tra) of the plasmid to a suitable recipient. During this process specific structural alterations are made to the host bacterial cell allowing attachment to the recipient and transfer of the plasmid DNA, and the bacterial chromosome if the plasmid should be integrated into it (Willets & Wilkins, 1984).

It is also possible for a conjugative plasmid to effect the mobilization and transfer of other, not normally conjugative plasmids, which coexist within the donor bacterium.

The plasmids of Bacteroides species have been investigated and several have been described (Guiney & Davis, 1975; Mays & Johnson, 1979; Wallace et al., 1981; Callihan et al., 1983; Zöllner et al., 1983). These plasmids were all cryptic and varied in size from 3 to 150 kb. The presence of the same plasmid in more than one species of Bacteroides has been shown by Mays & Johnson (1979) who used DNA-DNA reassociation to determine the homolgy between plasmids from four strains of Bacteroides and the plasmids extracted from 23 other plasmid-bearing strains of Bacteroides.

A 4.4 kb plasmid originally isolated from B.uniformis T1-1 was found in 15 of these strains. Callihan et al. (1983) were able to classify the plasmids of less than 8 kb from 15 isolates of Bacteroides into three DNA homology classes. Salyers (1984) suggests that these small plasmids may be parasites which encode only the functions needed for their own replication, but does not rule out the possibility that these plasmids have some ecological significance.

The resistance plasmids which have been reported to carry the clindamycin-erythromycin resistance determinant on a transposon Tn4400 have been discussed earlier in this chapter. The conjugal transfer of the plasmid pBFTM10 was enhanced by pretreatment of the donor with tetracycline but not clindamycin prior to mating (Tally et al., 1982). This did not apply to retransfer from the recipient to another Bacteroides strain, suggesting the involvement of chromosomal genes of the original donor in the transfer of the plasmid (Malamy & Tally, 1981).

The transfer of tetracycline resistance in Bacteroides seems to be effected in a novel way which has not been completely explained. The strain of B.fragilis which harbours the pBF4 Cc^r plasmid can also transfer tetracycline resistance to other bacteria (Smith et al., 1982) independently of the transfer of Cln^r, and no plasmid is associated with its transfer. Both resistance to tetracycline and the transfer of tetracycline resistance can be enhanced by pretreatment of the donor strain with sub-inhibitory concentrations of tetracycline. This phenomenon has also been noted in other strains of Bacteroides (Malamy & Tally, 1981).

The conjugal transfer of both tetracycline and clindamycin resistance by a strain of B.fragilis (V503) which contains a plasmid, to a plasmidless strain of B.uniformis (V528) has been described (Mays et al., 1982). Although the plasmid could be detected in the majority of resistant transconjugants, 20% of the resistant progeny were plasmid free. Using radio-labelled DNA probes they were unable to detect integration of the plasmid, or parts of the plasmid into the recipient chromosomal DNA. No other plasmid could be detected in these bacteria, but even so they were able to transfer the resistance determinants to suitable recipients in filter-mating experiments. They reported evidence which suggests that there was homology between chromosomal DNA and the Cc^r transposon, Tn4400. Similar results have been reported by Tally et al. (1981).

The Cc^r transposon, Tn4400, has also been reported to carry a resistance determinant against tetracycline, this is not expressed in Bacteroides or in E.coli grown anaerobically (Guiney et al., 1984a; 1984b; 1984c). During aerobic growth of E.coli this resistance is expressed. This tetracycline resistance determinant is not homologous with the tetracycline resistance determinants on the E.coli R factors R100, RK2, or pBR322 or with the conjugative tetracycline resistance of B.fragilis V479.

The transfer of antibiotic resistance between Bacteroides strains and E.coli has been reviewed by Salyers (1984) who finds that all reports of resistance transfer are not reliable and that further work needs to be done with the Bacteroides isolates that have been reported to conjugate with E.coli.

The transfer of hybrid Bacteroides plasmids by their mobilization by other conjugal plasmids to and from B.fragilis and E.coli, has been demonstrated by Guiney et al. (1984c), Shoemaker et al. (1985) and Robillard et al. (1985).

Guiney et al (1984c) have constructed a shuttle plasmid which was able to transfer genetic material between E.coli and B.fragilis. This plasmid (pDP1) contains the pBR322 replicon and the Bacteroides clindamycin resistance plasmid pCP1 linked to the transfer origin of the broad host range plasmid RK2. The helper plasmid pRK231 was used in a filter mating procedure to mobilize this recombinant plasmid (pDP1) from E.coli to B.fragilis with a frequency of approximately 10^{-6}. The helper plasmid was not maintained in the B.fragilis transconjugants. Using this system, they were able to demonstrate that antibiotic resistance genes active in B.fragilis were not active in E.coli and vice versa.

Shoemaker et al (1985) and Robillard et al (1985) working with the B.fragilis R plasmids, pBF4 and pBFTM10 respectively, showed that recombinants of these plasmids and E.coli conjugative plasmids could be mobilized into B.fragilis and could express the clindamycin resistance gene of the Bacteroides plasmids. This clindamycin resistance gene was shown by these methods to be situated on an active transposon-like structure, Tn4400, which contains active insertion sequence elements at its ends.

Smith, (1985a) reported a polyethylene glycol facilitated transformation system for B.fragilis plasmid DNA and the construction of chimaeric plasmids able to replicate in E.coli and B.fragilis. These plasmids have been constructed from a small (2.7 kb) cryptic Bacteroides plasmid (pBI143) and the E.coli cloning vector pUC19. The clindamycin resistance determinant from the Bacteroides R plasmid pBF4 has been included, allowing selection of Bacteroides transformants. These plasmids contain at least 10 single restriction endonuclease sites, suitable for cloning of DNA, and were shown to be replicated in E.coli and B.fragilis following transformation and could be selected by conferring resistance to ampicillin and clindamycin in their respective hosts.
These developments will allow the analysis of expression of E.coli genes in B.fragilis and vice versa.

However there is so far no evidence of the expression of an antibiotic determinant in both E.coli and B.fragilis.
This differential expression of genes between Bacteroides and E.coli raises the question of whether it will be possible to analyse the genes of Bacteroides by cloning into E.coli and complementation of defined mutants.

BACTERIOCINS

The bacteriocins (those produced by or effective against E.coli are known as colicins) have proved to be a useful tool in the analysis of the genetic mechanisms of plasmids (reviewed by Hardy, 1975; and Konisky, 1982).

Bacteriocins confer a competitive advantage to the producer bacterium under appropriate circumstances (reviewed by Israil, 1983). Hoyte & Sizemore (1982) studied a bacteriocin producing Vibrio harveyi strain and found that in simulated free-living conditions there was no competitive advantage attributable to bacteriocin production, but that in a simulated enteric habitat the producer strain achieved a 90% inhibition of sensitive strains. Similar results were obtained by Jorgensen et al. (1983) with strains of E.coli.

Olson & Means (1981) tested samples of drinking water and found that substances resembling bacteriocins could be detected in 20% of samples which were active against selected E.coli indicator strains.

Bacteriocins have been employed to advantage to protect plants from pathogenic Pseudomonas solanacearum by pretreatment of seedlings with a bacteriocin producer (Chen & Echandi, 1984). There has also been a report of neoplastic cell death following treatment with a purified bacteriocin from E.coli HSC10 (Farkas-Himsley & Yu, 1985).

Colicins have been used as a means of typing closely related isolates of pathogenic E.coli (Pugsley, 1985) and Salmonella agona (Viconta & Almeida, 1984).

The biology of colicins is well understood (Lazdunski et al., 1984). They are encoded by a plasmid which also carries the genes which control the production of the colicin. These genes are in turn repressed by the lexA protein, the repressor of the E.coli "SOS" DNA repair system (van den Elzen et al., 1982). The lexA control is released following exposure of the cell to DNA damaging agents such as UV irradiation, mitomycin C, or aging, and the active colicin is released. The plasmid also encodes an immunity protein which protects the producer strain from the effects of the colicin.

The modes of action of the colicins have been reviewed by Konisky (1982) and although they are varied, four main groups can be discerned.

1. The nucleases: These (Col E2, E3 & cloacin DF13) can traverse the cell membrane and degrade cellular DNA or RNA.
2. Membrane damage: Col A and several others cause the formation of ion permeable channels in the sensitive cell membrane. This results in the collapse of the membrane proton motive force.
3. Active transport inhibition: Col L acts by reducing the ATP driven active transport of the cell.
4. Cell wall damage: Col M causes cell lysis by the inhibition of murein synthesis and the promotion of murein hydrolysis.

Colicinogenic plasmids may also encode other functions such as serum resistance (Nilius & Savage, 1984) or virulence (Jones et al., 1982).

The evolution of the colicin genes is discussed by Hardy (1975) and because of extensive parallels with the biology of bacteriophage it is suggested that a bacteriophage would seem the most likely colicin ancestor in view of the action of bacteriphage ghosts, specific immunity and the similar effects of UV irradiation on lysogenic and colicinogenic bacteria.

Further evidence on this is provided by Steensma (1981) who investigated the killing of sensitive Bacillus subtilus cells by a defective bacteriophage. He found that cell death was caused by cell membrane leakage and the loss of the cell membrane potential, apparently similar to that caused by ColA. The active component was identified as a protein of approximate M_r 85000, which is similar to the size of many colicins. Whether this possible source of active bacteriocin genes applies to the non-plasmid borne bacteriocins of other bacterial species is not known.

Bacteria other than E.coli have bacteriocinogenic factors which are analogous to the colicinogenic factors of the Enterobacteriaceae, amongst which are the lactic Streptococci (Neve et al., 1984) and Streptococcus cremoris (Davey, 1984).

In many other species (B.fragilis, Streptococcus mutans, Myxococcus coralloides-D, Corynebacterium glutamicum, Neisseria meningitidis) the association of a bacteriocin with a plasmid has not been established (Mossie et al., 1979; Jyssum & Allunans, 1984; Karabekov et al., 1984; Munoz & Arias, 1984; Takada et al., 1984).

Bacteriocins have been reported from Bacteroides species. Beerens et al. (1966) and Podhaisky & Reingold (1970) found that bacteriocin production was common amongst strains which they had examined. Booth et al. (1977) investigated bacteriocin production by strains of Bacteroides isolated from human faeces from an individual involved in the American space program. Although four bacteriocin producing strains were isolated, only that produced by the T1-1 strain was partially characterized. These strains were found to co-exist in the colon with a larger population of non-bacteriocin producing, bacteriocin susceptible strains of Bacteroides. Austin-Prather & Booth (1984) report that the T1-1 strain has been identified as B.uniformis T1-1 and that the bacteriocin, which had earlier been reported to have an M_r of approximately 300000 could be purified in the presence of 6M guanidine hydrochloride or 7M urea and had an apparent M_r of 5000-6200.

The bacteriocin reported by Mossie et al. (1979) will be discussed in Chapter 2.

Hayes et al. (1983) reported the purification and characterization of a bacteriocin from B.fragilis 1356. This bacteriocin was extracted from the culture medium as a complex of protein, lipid and carbohydrate with an M_r of greater than 10^7. The complex was dissociated with 6M guanidine hydrochloride and further purification by gel-filtration on Sepharose 6B yielded a pure polypeptide with an M_r of approximately 5000. The mode of action of this bacteriocin on the indicator B.vulgatus 98-3 was not reported.

The strains of B.fragilis used in this study, BF-1 and BF-2, were isolated in 1977 from clinical infections and the species established by normal laboratory practice. Confirmation of the species of these strains was carried out by Professor L.V.Holdeman, Virginia Polytechnic Institute and State University, Anaerobe laboratory, Virginia, USA.

The strains were stored on slopes of complex medium at room temperature under anaerobic conditions, until 1981 when aliquots of early log phase cultures were stored frozen at -90°C, under anaerobic conditions. The original BF-1 strain was found to produce a bacteriocin (Mossie et al., 1979), for which the BF-2 strain was the sensitive indicator. The BF-2 strain was also used for studies of the effects of UV irradiation and oxygen on macromolecular synthesis and DNA repair (Jones et al., 1980; Jones & Woods, 1981; Schumann et al., 1982, 1984; Slade et al., 1981, 1982, 1983a, 1983b, 1984; Abratt et al., 1985, 1986).

AIMS OF THIS STUDY

This study aimed at providing a means for probing the molecular genetic organization of B.fragilis, particularly those strains where the DNA repair mechanisms had been described.

The following routes of investigation were followed; the bacteriocin of B.fragilis BF-1; the investigation of any plasmids which might be discovered, with the aim of constructing a hybrid plasmid which might replicate in both E.coli and B.fragilis; and the preparation of a genetic library which could be screened for Bacteroides genes which might function in E.coli.

Should any genes be isolated by screening the library they were to be studied with regard to their expression and regulation in E.coli.

CHAPTER 2.

A BACTERIOCIN FROM BACTEROIDES FRAGILIS BF-1.

SUMMARY

A cell bound bacteriocin was extracted from the cells of B.fragilis BF-1 by tris-buffer or osmotic shock. The purified bacteriocin was a protein with an approximate M_r of 7000, and was relatively heat stable. There was a delay of approximately 3.5h before DNA, RNA and protein synthesis were inhibited by the bacteriocin, this coincided with lysis of the susceptible indicator strain.

2. 1: INTRODUCTION.

Bacteriocin types and mode of action.

Bacteriocins and colicins have been isolated from most bacterial species which have been investigated. They are proteins and their molecular sizes (M_r) and modes of action vary widely. The known properties of some representative bacteriocins are summarized in Table 2. 1. Bacteria may also produce antibiotic-like substances (Rowe & Baron, 1981; Nakamura et al., 1983; Kader & Sahl, 1984) which have activity against a wide range of bacterial species but bacteriocins are characterized by a narrow range of specificity and sensitive strains are usually closely related to the producer strain (Konisky, 1982).

The presence of a bacteriocin in the BF-1 strain of B.fragilis was first reported by Mossie (1979) and further investigations by Mossie et al, (1979,1980,1981) indicated that this bacteriocin was unique in its mode of action compared with other bacteriocins (Reviewed by Konisky, 1982). The mode of action postulated was the inhibition of the action of ribonucleic acid polymerase (RNA-polymerase), the enzyme required for the transcription of functional messenger RNA in the sensitive bacteria. Bacteriocins have been isolated from other species of Bacteroides, but none had properties which resembled those described by Mossie et al. (1980).

When the BF-1 strain was investigated during this study, a bacteriocin was isolated which did not exhibit the properties described by Mossie et al. (1979 & 1980). Culture supernatants were still able to inhibit the growth of the indicator strain, but the properties differed.
To simplify the description of the bacteriocins, that described by Mossie et al (1979) is designated K-BF-1; while that described in this thesis and by Southern et al (1984), is designated J-BF-1.

The production of more than one bacteriocin has been noted in the Enterobacteriaceae (Tagg et al., 1976; Cooper & James, 1984) so that it was possible that the loss of a plasmid coded bacteriocin accounted for the apparent change in the properties of the inhibitory substance produced by the BF-1 strain of Bacteroides fragilis.

In order to elucidate these differences the inhibitory substance produced by the BF-1 strain was investigated in more detail.

TABLE 2.1. SUMMARY OF SOME BACTERIOCIN TYPES AND MODES OF ACTION

BACTERIOCIN NAME	Mol. size Mr	Mode of action	reference
ACNECIN (Propionibacterium acnes)	12 000	Bacteriostatic	Fujimura & Nakamura (1978)
BACTERIOCIN 28 (Clostridium perfringens)	84 000	Not known	Li et al. (1982)
BUTYRICIN 7423 (Clostridium butyricum)	32 500	ATP-ase	Konisky (1982)
LACTACIN B (Lactobacillus acidophilus)	6 500	Bactericidal	Narefoot & Klaenhamer (1984)
MEGACIN A-216 (Bacillus megaterium)	51 000	Membrane damage	Konisky (1982)
PESTICIN A1122 (Yersinia pestis)	65 000	Cell-wall synthesis inhibition	Hu & Brubaker (1974) Ferber & Brubaker (1979)
PYOCIN AP41 (Pseudomonas pyocyaneus)	90 000*	DNA-endonuclease	Konisky (1982)
STAPHYLOCOCCIN 1580 (Staphylococcus epidermidis)	20 000*	Cell-membrane Ion-leakage	Jetten et al. (1972a)(1972b)
COLICIN L (Serratia marcescens)	64 000	Active-transport inhibition	Foulds (1971)(1972)

TABLE 2.1. (continued)

BACTERIOCIN NAME	Mol. size Mr	Mode of action	reference
Escherichia coli:			
COLICIN A	63 000	Cell-membrane ion-leakage	Morlon et al. (1983)
COLICIN M	27 000	Cell-wall synthesis inhibition	Schaller et al. (1981)(1982)
COLICIN 1B	71 000	Cell membrane ion-leakage	Varley & Boulnois (1984)
COLICIN E-2	50 000*	DNA-membrane complex damage	Beppu & Arima (1972)
COLICIN E-3	50 000	RNA-nuclease	Konisky (1982)
COLICIN K	45 000	Cell membrane ion-leakage	Konisky (1982)
COLICIN V	4 000		Frick et al. (1981)
K-BF-1 (Bacteroides fragilis BF-1)	16 000	RNA-polymerase inhibition	Mossie et al. (1979,1980,1981)
T1-1 (Bacteroides uniformis T1-1)	6 000*	Not known	Booth et al. (1977) Austin-Prather & Booth (1984)
b-1356 (B.fragilis 1356)	5 000*	Not known	Hayes et al. (1983)
MELANINOCIN (Bacteroides melaninogenicus)	Not known	Not known	Nakamura et al. (1981)

* = Active unit.

2. 2. MATERIALS AND METHODS

2. 2. 1: Bacterial strains:

Bacterioides fragilis strains were obtained from the culture collection at The University of Cape Town Department of Microbiology . The bacteriocin producer strain BF-1 and the indicator strain, BF-2 have been described by Mossie et al. (1979).

2. 2. 2: MEDIA FOR BACTERIAL GROWTH:

The complex brain-heart infusion (BHI) medium described by Mossie et al.(1979) was used initially but because of buffering problems a more reliable growth was obtained using a medium based on the US Pharmacopoeia Fluid Thioglycollate medium but without the agar or sodium thioglycollate. This was called Bacteroides broth (Southern et al., 1984). Complete descriptions of the constituents and methods of manufacture of all media are given in appendix 2.

2.2.3 BUFFERS, ELECTROPHORESIS AND SOLUTIONS:

The formulations for the buffers used in this section are given in appendix 3. Electrophoretic techniques are described in Appendix 4.

2. 2. 4. ASSAY OF BACTERIOCIN:

Dilutions of the bacteriocin were prepared in tris-buffered saline; pH7.4 (TMS) using siliconized polystyrene micro-titre trays. Porcelain fish-spine beads (Richardson, 1968) were charged with these dilutions (about 10ul per bead) and applied to the surface of a BHI plate covered with 3ml of BHI soft agar seeded with 0.1ml of a fresh log phase culture of the indicator (BF-2) strain of B. fragilis. After overnight anaerobic incubation, zones of inhibition were noted and the titre determined. The titre was expressed as the reciprocal of the dilution giving a visible zone of inhibition as arbitrary units (AU) per 10ul.

On occasions the titre was estimated from the size of the zone of inhibition produced by a single sample. The zone diameter was read from a graph of zone size vs AU which had been produced from titrations of some 120 samples.

Protein was determined by the dye-binding method of Bradford (1976).

2. 2. 5. LOCALIZATION OF BACTERIOCIN PRODUCTION.

The localization of the cell-bound bacteriocin was investigated by treating the cells harvested from a 16h culture in Bacteroides broth, in various ways. These included 1M NaCl, 20%(w/v) sucrose-2mM EDTA, 20%(w/v) sucrose-2mM EDTA followed by distilled water, 0.01M tris-HCl (pH 8.0) followed by ultrasonication (U-S)(Heat Systems, USA), and U-S alone.

2. 2. 6. EXTRACTION OF BACTERIOCIN FROM B. fragilis BF-1:

Bacterial cells were collected from broth cultures by centrifugation and resuspended in one tenth the culture volume of 0.01M tris-HCl buffer, ph 8.0, well mixed and kept at room temperature (18-25°C) for 30 min. The cells were removed by centrifugation and this crude extract was assayed for bacteriocin activity.

2. 2. 7. PRODUCTION OF BACTERIOCIN BY B.fragilis BF-1.

The production of bacteriocin by a culture of B.fragilis BF-1 was monitored as follows. One litre of Bacteroides broth was inoculated with 10ml of an overnight culture of the BF-1 strain and incubated anaerobically at 37°C. Samples were withdrawn at intervals, without introducing air to the culture. The absorbance of the sample at 600nm was determined. Cells were collected from 10ml of the sample in tared conical centrifuge tubes and the wet weight of the bacteria determined. These cells were then extracted with 1.0ml of 0.01M tris-HCl buffer (pH 8.0) for 30min and the supernatant collected and filter sterilized (0,22um Millex-GS; Millipore Corp.).

The bacteriocin content of the extract at each time interval was determined.

2. 2. 8. PURIFICATION OF BACTERIOCIN FROM CRUDE EXTRACTS:

The crude extract of bacteriocin was absorbed onto a column (25 by 200 mm) of freshly regenerated DEAE-cellulose (DE-52; Whatman, Inc.) equilibrated with 0,01M tris-HCl, pH8.0, washed with the same buffer containing 0,01M KCL, and the column developed with a linear gradient of 0.01M to 0.2M KCl in the same buffer. Fractions were collected and assayed for bacteriocin activity. Fractions showing bacteriocin activity were pooled, dialyzed (Spectropore 4-6000 MW cutoff) against distilled water for 6h, lyophilized, reconstituted in about 1/10 of the original volume, and loaded onto a Sephacryl S200 (Pharmacia Corp.) column (25 by 850mm) equilibrated with 0.1 M tris-HCl buffer, pH8.0, containing 0.5M NaCl. This buffer was used to elute 5ml fractions from the column at a flow rate of 30ml per hour. Active fractions were pooled, filter sterilized , and lyophilized.

Octyl-sepharose (Pharmacia Corp.) was regenerated according to the manufacturers instructions. This consisted of washing the gel with ethanol followed by equilibration in 50% saturated ammonium sulphate solution. The sample was diluted with an equal quantity of saturated ammonium sulphate and loaded onto the column. Fractions were collected as the column was developed at 0.5ml/min with a linear gradient of 50% saturated ammonium sulphate to 50% ethylene glycol. The fractions which showed bacteriocin activity were further analysed by SDS-PAGE.

2. 2. 9: STABILITY OF THE BACTERIOCIN.

a. Temperature stability: Triplicate samples of bacteriocin were sealed in glass ampoules and subjected to temperatures ranging from 4°C to 121°C for various times before titration for residual bacteriocin activity.

b. pH Stability: The effect of pH on the stability of the purified bacteriocin was determined in 0.1M citrate-phosphate-borate hydrochloride buffer (Diem & Lentner, 1975). A purified bacteriocin preparation was dialyzed against distilled water, and 10ul was added to 90ul of the various pH buffers in steps of one pH unit from pH 3 to 10. After 4h at 8 to 10°C, the samples were assayed for residual activity.

c. Enzyme Stability: The susceptibility of the bacteriocin to proteolytic enzymes was tested by incubation of a purified preparation of bacteriocin with Pronase E (Sigma) at a concentration of 1mg/ml (pH7.6) at 37°C for 30 min. Trypsin (Difco, 1:250), RNase and DNase (Miles Labs.) were also tested under the same conditions. After incubation, the mixtures were heated at 65°C for 20min to reduce any possible interference by the enzymes in the bacteriocin assay. This heat treatment reduced the activity of Pronase by 50% and trypsin by 90%. The sensitivity of B.fragilis BF-2 cells to the bacteriocin was not affected by pretreatment with the proteases, followed by washing in growth medium.

2. 2. 10: ASSAY FOR ALKALINE PHOSHATASE ACTIVITY:

Samples were diluted in 0.15M NaCl and 10uℓ was mixed with 100uℓ p-nitrophenyl phoshate (1mg/mℓ) in diethanolamine buffer (0.1M pH9.5) and incubated at 37°C for 30min. The production of a visible yellow colour was used as a qualitative indication of alkaline phosphatase activity.

2. 2. 11. ASSAY FOR RNA-POLYMERASE ACTIVITY AND ITS INHIBITION:

The method of Robb et al. (1977) was used for the assay of RNA-polymerase. Duplicate samples taken from various stages during the purification of the bacteriocin were added to the RNA-polymerase reaction mixtures and the reduction of [^{3}H]- uridine incorporation into trichloracetic acid precipitable material determined in relation to a buffer control. This was done using both partially purified E.coli and B.fragilis RNA-polymerase enzymes purified from the bacteria according to methods described in Appendix 5.

2. 2. 12. DEGRADATION OF RNA BY PURIFIED BACTERIOCIN.

[^{14}C]-RNA was prepared from E.coli C600, grown in minimal medium supplemented with casamino-acids (1g/l) and [^{14}C]-uracil (0.5μCi/ml), according to the method of Giraud (1967). The [^{14}C]-RNA was incubated with samples taken during bacteriocin purification and the residual trichloracetic acid precipitable label determined in relation to bacteriocin AU. Duplicate samples were tested and the averaged result reported.

2. 2. 13. METHODS OF PLASMID EXTRACTION:

The presence of plasmids in the bacteriocin producing B.fragilis BF-1 strain were not detected using the methods of Davis et al. (1980), Ish-Horowitz and Burke (1981), and Portnoy et al. (1981). In addition the following method used: Bacteria were collected from a 250ml 16h anaerobic culture, washed with 0,01M tris-HCl, pH 8,0, and resuspended in 5ml of 0,15M NaCl. To this suspension was added 10mg of Pronase E (E.Merck) and 100mg of SDS and gently mixed to dissolve the chemicals and lyse the cells. The lysate was gently extracted with phenol/chloroform/amyl alcohol (vols: 50/49/1). The nucleic acids were precipitated with an equal volume of isopropanol, the pellet washed with 70% ethanol, air dried and redissolved in 4ml of tris-EDTA (TE) buffer. The sample was electrophoresed on agarose as described in Appendix 4.

2. 2. 14: ATTEMPTS TO INDUCE BACTERIOCIN SYNTHESIS.

a. Mitomycin C (0.2 to 1.0µg/ml in steps of 0.2µg) was added to individual growing cultures of B.fragilis BF-1. The MIC for mitomycin C in B.fragilis BF-1 was about 1 µg ml^{-1}, although poor growth was obtained from 0.6 µg ml^{-1}. These cultures were sampled after 6 and 24h and the supernatant titrated for bacteriocin activity.

b. Separate cultures of B.fragilis BF-1 were exposed to 0.5, 1.0, 2.0, 4.0 and 16 Joules/m^2 from a germicidal UV lamp. The cultures were sampled after 6 and 24h anaerobic incubation and the supernatant titrated for bacteriocin activity.

2. 2. 15:

EFFECT OF BACTERIOCIN ON MACROMOLECULAR SYNTHESIS

Syntheses of DNA, RNA and protein by the indicator strain (BF-2) in the presence of bacteriocin were determined under strict anaerobic conditions by the incorporation of [2-^{14}C]-thymidine (5µg/ml, 3µCi/ml), [^{3}H]-uracil (20µg/ml, 15µCi/ml), and [^{35}S]-methionine (25µg/ml, 20µCi/ml), respectively, into cold trichloracetic acid-precipitable material. Exponential phase cells were labelled for 30min before the addition of purified bacteriocin (8 AU/ml).

2. 2. 16: MOLECULAR SIZE ESTIMATIONS.

The M_r of the purified bacteriocin was determined by gel-filtration chromatography on a calibrated column of Sephacryl-S200. The column was packed and eluted with the same buffer (0.1M tris-HCl, pH8.0 containing 0.5M NaCl). Fractions of the eluate were tested for bacteriocin activity and the peak of activity was used to extrapolate from the calibration curve of the column the M_r of the bacteriocin. This estimate was for undissociated, active bacteriocin.

SDS-PAGE was also used to estimate the M_r of the bacteriocin molecule. A 15%(w/v) polyacrylamide gel was used with a set of low molecular weight markers (Pierce, Lo-ranger).

2. 2. 17: IDENTITY OF THE PURIFIED PROTEIN AND BACTERIOCIN

To demonstrate that the protein band seen on SDS-PAGE was the bacteriocin, non-denaturing PAGE was undertaken using a phosphate buffer system (Andrews, 1981), after electrophoresis the gel was cut parallel to the electrophoresis lanes, one half was stained with coomassie blue and the other half was briefly washed with 0.01M tris-HCl, pH8, and laid onto the surface of a bacteriocin assay plate seeded with the sensitive indicator strain of Bacteroides and incubated anaerobically overnight. The plate was then examined and compared with the stained slice of gel.

2.2.18: ELECTRON MICROSCOPIC EXAMINATION OF BACTERIOCIN PREPARATIONS:

Both crude and purified preparations of bacteriocin were spread onto carbon coated grids, stained with sodium phosphotungstate and examined at a magnification of 50 000 for the presence of bacteriophage or phage-like particles.

2. 3. RESULTS

2. 3. 1: PRODUCTION OF THE BACTERIOCIN J-BF-1 BY B.FRAGILIS BF-1

2. 3. 1. 1: GROWTH OF B.FRAGILIS IN LIQUID CULTURES.

The growth medium which had been used by Mossie (1979) was formulated with 40mM of Na_2CO_3 to maintain a neutral pH in an atmosphere of H_2 and CO_2 (Moodie & Woods, 1973). As the available gas mixture was 85% N_2, 10% H_2 and 5% CO_2, the Na_2CO_3 caused alkaline conditions in the medium resulting in poor growth. A medium containing tryptone, yeast extract and cysteine (Bacteroides broth: Appendix 2) was found to give consistently good growth of all B.fragilis strains tested, in quantities of 25ml to 5000ml.

2. 3. 1. 2: THE ASSAY OF BACTERIOCIN J-BF-1

The titre of bacteriocin samples was readily determined using the dilution end point. The fish-spine beads were found to be more convenient and reproducible than punching wells in the agar.

The relationship between the area of the zone of inhibition of the sensitive indicator strain (BF-2) and the dilution end point of the bacteriocin showed a linear relationship. This relationship was employed as a semi-quantitative assay to determine the approximate titre of multiple samples such as gel chromatography fractions.

Determination of bacteriocin titres by this means was not entirely reliable, as had been noted by Mayr-Harting et al. (1972) due to the seeding rate of the indicator organism which had a significant effect on the size of the zone of inhibition caused by the bacteriocin. While every attempt was made to standardize the assay method using a control sample on each plate, variations did occur, so that for the reported experiments, except for column chromatography fractions, the dilution assay was carried out.

2. 3. 1. 3. PRODUCTION OF BACTERIOCIN IN LIQUID CULTURES:

The results are shown in Fig. 2. 1.

Under these conditions, the content of bacteriocin was proportional to the quantity of bacterial cells. The results also showed that the bacteriocin was associated with the bacterial cells. The quantity of bacteriocin in the supernatant medium remained low, but increased as the culture aged. Even in late stationary phase culture the free bacteriocin levels were not high. This pattern of bacteriocin production was similar in Fluid thioglycollate medium (Difco) and the brain heart infusion medium of Mossie et al. (1979).

The activity of more than one inhibitory substance could have given the results shown in Fig 2.1, but analysis of crude and purified cell extracts indicated that only a single inhibitor was present.

No bacteriophage or phage-like particles were seen in the electron micrographs.

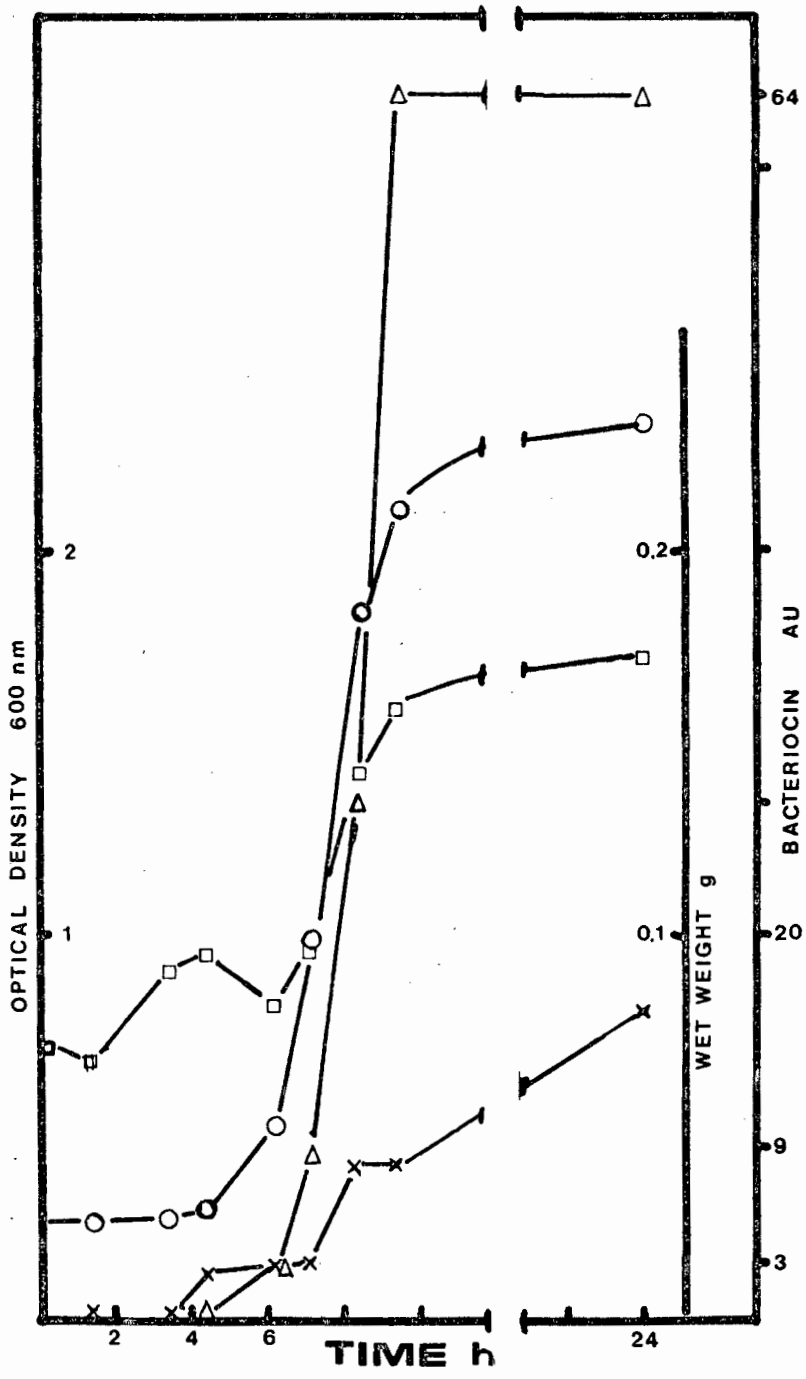


FIGURE 2. 1. Production of bacteriocin by a growing culture of B.fragilis BF-1 in Bacteroides broth. Extracellular bacteriocin (×), Cell-bound bacteriocin (△), Culture OD_{600} (○), Bacterial wet weight (□).

2. 3. 1. 4: LOCALIZATION OF BACTERIOCIN PRODUCTION.

The results of these experiments are shown in Table 2. 2. Bacteriocin activity was never detected in high concentration in the culture supernatant, and it was only by extracting the cells that good yields were obtained.

The bacteriocin was not released from the bacterial cells in any quantity by hypertonic or hyperosmotic treatments, but low molarity buffer or distilled water extracted a large amount of bacteriocin without appreciable cell lysis. U-S released bacteriocin from the lysed cells in similar quantities to that released by the low molarity buffer extraction.

The protein content of the buffer extract was low (0.29mg/ml) and unconcentrated extracts analysed by SDS-PAGE stained with coomassie blue appear almost devoid of protein (Fig. 2. 4 lane f); however silver staining (Biorad) of similar gels (Fig. 2. 5 lane a) revealed the presence of many proteins, albeit in small amounts. The U-S extracts, in contrast, show the presence of large quantities of different proteins.

The presence of the bacteriocin in the culture supernatant proved to be variable, on some occasions none was detectable and on others a titre of up to 16 AU was noted. Even when culture supernatants contained substantial amounts of bacteriocin activity, concentration and purification from this source was unsuccessful as the spent culture medium contained relatively large quantities of contaminating substances. The buffer extract was considered a more suitable substrate for further purification.

TABLE 2. 2. THE CELLULAR LOCATION, PURIFICATION AND SPECIFIC ACTIVITIES OF BACTERIOCIN J-BF-1 FROM BACTEROIDES FRAGILIS

PURIFICATION STEP	VOLUME (ml)	PROTEIN (ug/ml)	TOTAL PROTEIN (mg)	J-BF-1 ACTIVITY (AU/10ul)	TOTAL ACTIVITY (AU)	SPECIFIC ACTIVITY (AU/mg)
Culture supernatant	1800	10	18	2	-	-
NaCl (1M) extract	100	42	4.2	1	100	23.8
Sucrose-EDTA extract	60	10	0.6	2	120	-
Osmotic shock fluid	50	1400	70	32	1600	22.8
U-S extract after osmotic shock	50	2280	114	8	400	3.5
U-S extract of whole cells	50	3660	183	32	1600	8.6
Tris-HCl (0.01M) ext.	50	290	14.5	32	1600	110.3
U-S extract after tris-HCl extract	25	3550	88.8	16	400	4.5
DE-52 activity peak pool	38	83	3.2	16	608	192
S-200 activity peak pool	11	8	0.09	32	352	3911

RNA-polymerase, a known cytosolic enzyme was not detected in Tris-buffer or sucrose/distilled water extracts of cells which produced bacteriocin, either as active enzyme or as the characteristic beta and beta prime polypeptide subunits on SDS-PAGE.

2. 3. 1. 5. ELECTRON MICROSCOPIC EXAMINATION OF BACTERIOCIN

No phage-like particles were seen in electron micrographs of crude or purified preparations of bacteriocin at a magnification of 50000.

2. 3. 2. PURIFICATION OF THE BACTERIOCIN J-BF-1

The purification of the bacteriocin from B.fragilis BF-1 by DEAE-cellulose and Sephacryl S-200 chromatography is shown in Figs. 2. 2 and 2. 3, and Table 2. 3. Analysis of samples from these steps by SDS-PAGE is shown in Fig. 2. 4 and Fig. 2. 5.

Additional purification on Octyl-separose did not show an increase in purity, while a large proportion of activity was lost. Purification using DEAE-Sephadex (Pharmacia) showed no improvement over the more economical DEAE-cellulose. One of the contaminants of the buffer extract of the cells was a significant quantity of alkaline phosphatase activity. This co-purified with the bacteriocin activity after DEAE-cellulose chromatography but was effectively separated from it by Sephacryl S-200 chromatography.

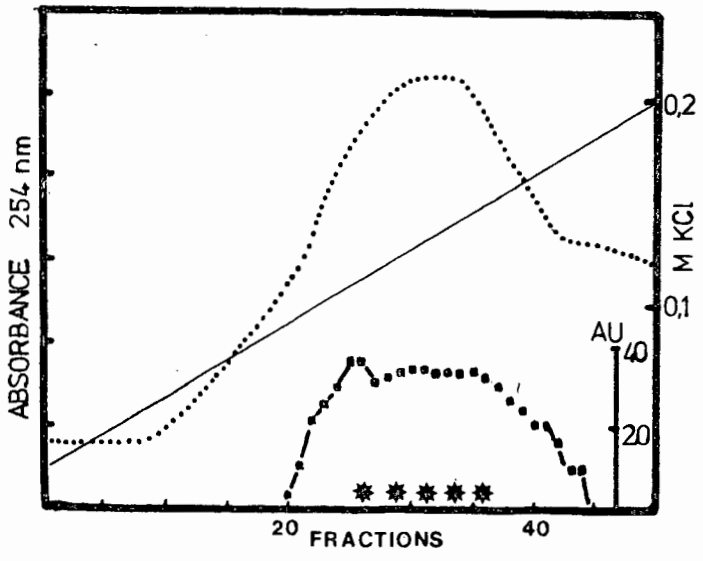


FIGURE 2. 2. DEAE-Cellulose purification of bacteriocin J-BF-1. Percent absorbance at 254nm (•••), samples showing alkaline phosphatase activity (✸), KCl concentration (——), and bacteriocin activity (▪).

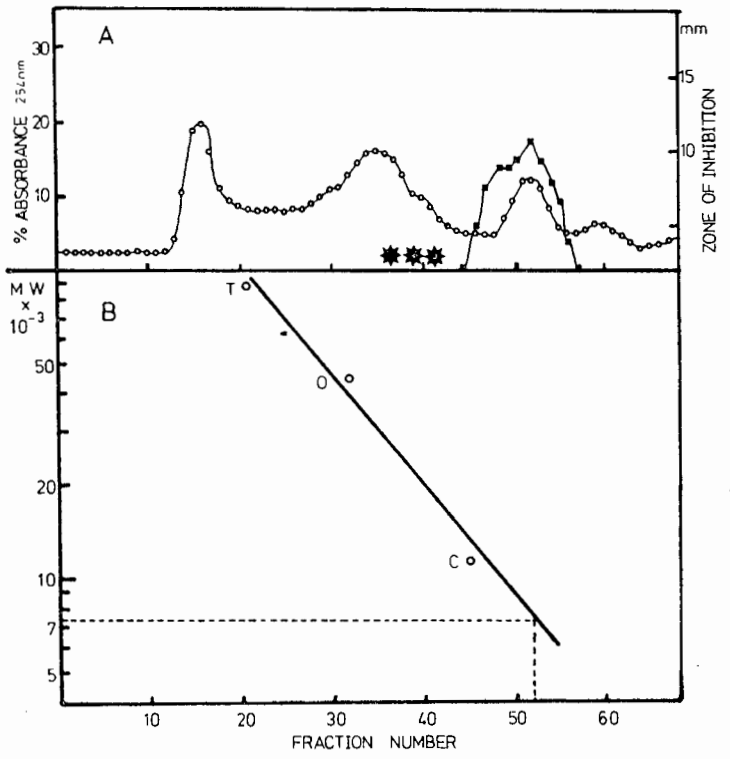


FIGURE 2. 3. Sephacryl S-200 gel filtration chromatography. A, purification a concentrated sample of J-BF-1 from DEAE-cellulose ; Percent absorbance at 254nm (∘), alkaline phosphatase (✸) and bacteriocin activity (▪). B, Molecular size estimation using transferrin (T, 90000), ovalbumin (O, 45000), and cytochrome c (C, 12400). The flow rate of the column was 10ml/h.

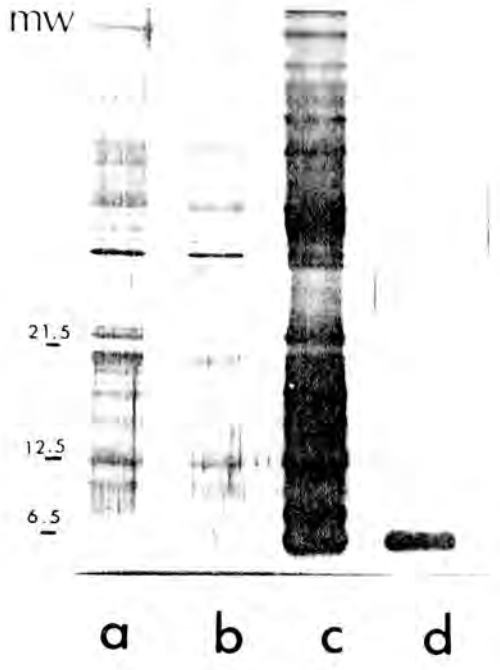


FIGURE 2. 5. SDS-PAGE of extracts before and after purification by DEAE-cellulose and Sephacryl S200 chromatography. Lanes a. Tris (0.01M) extract of the B.fragilis BF-2 indicator strain; b. Tris (0.01M) extract of the BF-1 producer strain ; c. Fraction showing bacteriocin activity after DEAE-cellulose chromatography; d.Fraction showing bacteriocin activity after Sephacryl S200 chromatography. MW, molecular weight (X 10^3) markers; aprotinin (6.5), cytochrome C (12.5) and soybean trypsin inhibitor (21.5). Stained with Biorad silver stain. The lowest band (clearly visible in lane d) was only seen in fractions which showed bacteriocin activity.

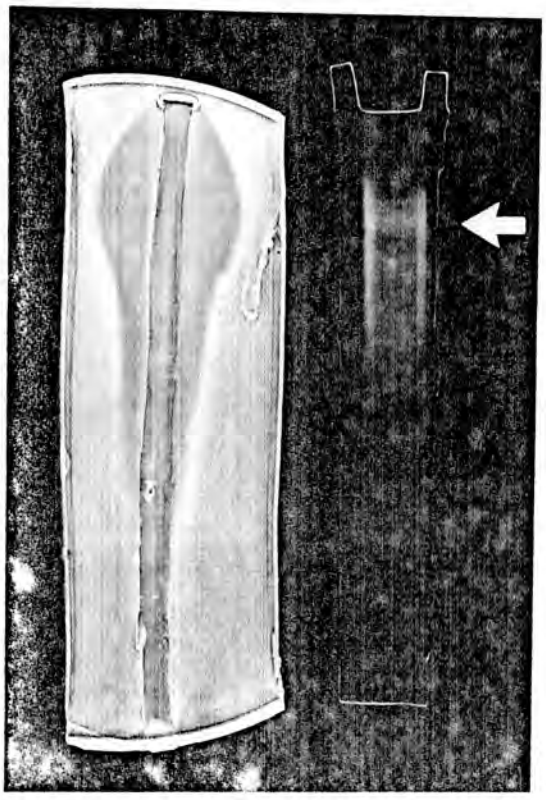


FIGURE 2. 6. Non-denaturing polyacrylamide gel electrophoresis of bacteriocin and demonstration of the active protein. A, Slice of an electrophoresis gel, loaded with concentrated purified bacteriocin which has been laid onto the surface of a bacteriocin assay plate and incubated anaerobically to show the inhibition of growth of the indicator strain.; B, a similar slice which has been stained with coomassie blue R250. The arrow indicates the zone of inhibition and the diffuse protein band.

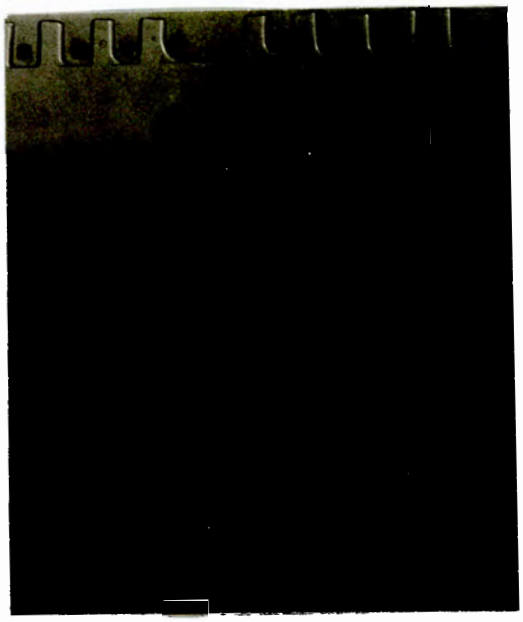


FIGURE 2. 4. Bacterial extracts analyzed by SDS-polyacrylamide gradient electrophoresis and stained with coomassie blue R250. Lane a. Purified and concentrated bacteriocin; b, culture supernatant; c, 20% sucrose-2mM EDTA extract; d, osmotic shock fluid after sucrose; e, extract of U-S whole cells; f, 0.01M Tris extract; g, supernatant from U-S lysate of osmotically shocked (sucrose) cells; h, extract of U-S cells after 0.01M Tris extraction; m, marker proteins; ovalbumin, human growth hormone, cytochrome C (bovine), and insulin B chain ($\underline{M}_r$ X 10^3). Equal volumes of the cell extracts described in Table 2. 3 were applied to lanes b through h.

2. 3. 3: PROPERTIES OF THE BACTERIOCIN J-BF-1.

2. 3. 3. 1. STABILITY OF THE PURIFIED BACTERIOCIN.

pH: The bacteriocin was stable between pH6 and pH9. There was a marked reduction in activity above pH9 and some reduction in activity below pH6 (Table 2. 4).

Temperature: The bacteriocin activity was stable at 50°C and 60°C for 5h and 3h respectively. At 70°C there was an initial 50% loss of activity after 1h but no further loss in activity over 4h. After autoclaving at 121°C for 15 min, 25% of the initial activity was retained. No loss of activity of samples stored at 4 - 10°C for 2 years was detected (Table 2. 4.).

Enzymes: The activity of the purified bacteriocin was not reduced by the enzymes RNase or DNase, but was substantially reduced by treatment with Streptomyces griseus protease, and partly by treatment with trypsin. These results are shown in Table 2. 4.

TABLE 2. 3.

STABILITY OF BACTERIOCIN J-BF-1

Details of the treatment conditions are described in Matetials and Methods section, 2. 2. 9.

TREATMENT CONDITIONS	RESULTS
	Residual activity(%)
pH 5	75
pH 6 - pH 9	100
pH10	25
4 - 10°C for up to 24 months	100
18 - 20°C for up to 3 days	100
50°C for 5h	100
60°C for 3h	100
70°C for 1h	50
70°C for 3h	50
121°C for 15min	25
DNase (1mg/ml) for 1h	100
RNase (1mg/ml) for 1h	100
Pronase E (1mg/ml) for 30min	20
Trypsin (1mg/ml) for 30min	80

2. 3. 3. 2. MOLECULAR SIZE OF THE PURIFIED BACTERIOCIN.

The position of the peak of bacteriocin activity from the calibrated S200 column was used to estimate the M_r of approximately 7200. This estimate was for undissociated, active bacteriocin (Fig. 2. 3B).

SDS-PAGE was also used to estimate the M_r of the bacteriocin molecule. A single band was visible in purified preparations which was estimated to have an approximate M_r of about 6400 and was present in all preparations which had bacteriocin activity, Fig. 2. 4 and Fig. 2. 5.

Although non-denaturing PAGE did not resolve the proteins as sharply as SDS-PAGE, this test showed that the only protein band in the purified preparation was associated with bacteriocin activity, Fig. 2. 6.

The activity was retained by dialysis tubing with a nominal molecular weight cut-off of 6-8000, but was gradually lost from dialysis tubing with a 12-14000 nominal molecular weight cut-off (Spectropor).

There was no detectable bacteriocin in higher M_r fractions after Sephacryl-S200 chromatography, indicating the absence of active polymers or aggregates of the bacteriocin.

2. 3. 3. 3. SPECIFICITY OF BACTERIOCIN J-BF-1.

Assay plates were prepared, seeded with each of 32 Bacteroides strains from the culture collection of the University of Cape Town, Department of Microbiology, which had been isolated from clinical material and had been shown to differ with respect to their susceptibility to various

phages and bacteriocins. Of these, eight were sensitive to bacteriocin J-BF-1.

Clinical isolates of Escherichia, Klebsiella, Acinetobacter, Pseudomonas and Staphylococcus were also tested. None of the strains were sensitive to the bacteriocin J-BF-1.

Mossie et al. (1980), found that the bacteriocin K-BF-1 inhibited the action of RNA-polymerase, and isolated mutants of the B.fragilis BF-2 indicator strain which were resistant to the action of rifampicin. The mode of action of this antibiotic is to inhibit bacterial RNA-polymerase (Schultz & Zillig, 1981) and as these mutants were also found to be resistant to K-BF-1, this was used as evidence for its mode of action. These mutants were used to prepare assay plates, but although they were resistant to levels of rifampicin which inhibited the wild type indicator strain they were sensitive to the bacteriocin J-BF-1.

It was noted that if assay plates spread with the indicator B.fragilis BF-2 strain were incubated anaerobically for over 48h, colonies appeared within the inhibitory zones caused by the bacteriocin. It was determined that about 10^{-5} of the total population were resistant even when the indicator culture had been freshly cloned from a single colony. These resistant colonies, when subcultured, proved to be stable and characteristic of B.fragilis BF-2. They still contained the pBF-C1 plasmid described in chapter 3. These bacteriocin resistant clones proved to be equally sensitive to rifampicin as was the parent BF-2 strain.

2. 3. 4. MODE OF ACTION OF BACTERIOCIN J-BF-1.

2. 3. 4. 1. Inhibition of RNA-polymerase:

The enzyme RNA-polymerase was partially purified from E.coli and more completely purified from B.fragilis BF-2 as described in Appendix 5. These preparations were active in the in vitro assay of Robb et al. (1977), which had been used to reveal the inhibitory activity of the K-BF-1 bacteriocin (Mossie et al., 1980).

When samples of bacteriocin from various stages of purification were tested for the ability to inhibit this in vitro assay, it was found that as the purity of the bacteriocin preparation increased there was a concomitant decrease in the ability of the preparation to inhibit the synthesis of RNA (Table 2. 4).

Alkaline phosphatase: It was considered possible that the alkaline phosphatase activity which co-purifies with the bacteriocin from DEAE-cellulose might have been the active inhibitory principle in the RNA-polymerase assay, perhaps by degrading the substrate nucleotide triphosphates or the end product of the enzyme reaction, single-stranded RNA. However it was shown that the bacteriocin and the alkaline phosphatase activity could be separated from the RNA-polymerase inhibitory activity on Sephacryl S-200 (Table 2. 4).

Similarly, more purified fractions of bacteriocin J-BF-1 were found to cause less degradation of [^{14}C]-RNA than crude material (Table 2. 5).

TABLE 2. 4.

. INHIBITION OF PARTIALLY PURIFIED RNA-POLYMERASES.

Fractions (10µℓ) were added to the RNA-polymerase assay mix and the percentage reduction of label incorporation calculated in relation to a control to which 10µℓ of 0.15M NaCl had been added.

EC=RNA-polymerase from E.coli.

BF=RNA-polymerase from B.fragilis.

Inhibitory substance:	Percent inhibition	
	EC	BF
Buffer Control (100%)	0	0
Bacteriocin peak fraction from DEAE-cellulose	85	82
Bacteriocin peak fraction from S-200	2	7
Alkaline phosphatase activity from S-200	0	not tested
Rifampin 0.3 µg ml^{-1}	97	99

TABLE 2. 5.

DEGRADATION OF RADIOLABELLED RNA BY BACTERIOCIN

Samples (10µℓ) were incubated for 30 min with 100µℓ of [^{14}C]-RNA (5700cpm) at 37°C. Material precipitated by cold 5% TCA was collected and counted in a liquid scintillation counter and the residual percentage counts calculated relative to a 0.15M NaCl control. The specific degradation by the bacteriocin was calculated as percentage degraded per AU bacteriocin.

SAMPLE	J-BF-1	RESIDUAL	SPECIFIC DEGRADATION
	AU	%	%/AU
Control	-	100	-
Crude extract	16	35	4.1
DE-52 peak	32	36	2.0
S-200 peak	64	74	0.4
RNase (1mg/ml)	-	7	-

As the labelled RNA preparation consisted largely of stable ribosomal RNA with extensive secondary structure, it is possible that the bacteriocin could have an effect on mRNA which would not be detectable by this assay.

2. 3. 4. 2.

EFFECT OF BACTERIOCIN ON MACROMOLECULAR SYNTHESIS AND CELL GROWTH

The uptake of radio-labelled thymidine, uracil and methionine into DNA, RNA and protein respectively, by a growing culture of the sensitive indicator strain in the presence of bacteriocin is shown in Fig. 2. 7. This figure also indicates the effect of the bacteriocin on cell growth as measured by optical density.

There was no detectable difference between the control and treated cultures until 4h had elapsed. This effect was reproducible as the test was carried out three times with essentially similar results. Due to the time course of bacteriocin action, this experiment measures the effects on stable ribosomal RNA and effects of the bacteriocin on mRNA might be revealed by pulse labelling experiments.

There was no clear cut time difference in the inhibition of DNA, RNA, protein synthesis or cell growth. From the curve of label incorporation a doubling time of about 50min can be estimated, thus the cells must have undergone at least four divisions in the presence of bacteriocin before the effects became evident. The decrease in optical density may have been due to cell lysis or changes in cell stucture. The bacteriocin was only active during active growth of the sensitve bacteria as a drop of bacteriocin placed onto an already grown lawn of indicator strain on BHI agar did not cause detectable lysis after overnight anaerobic incubation.

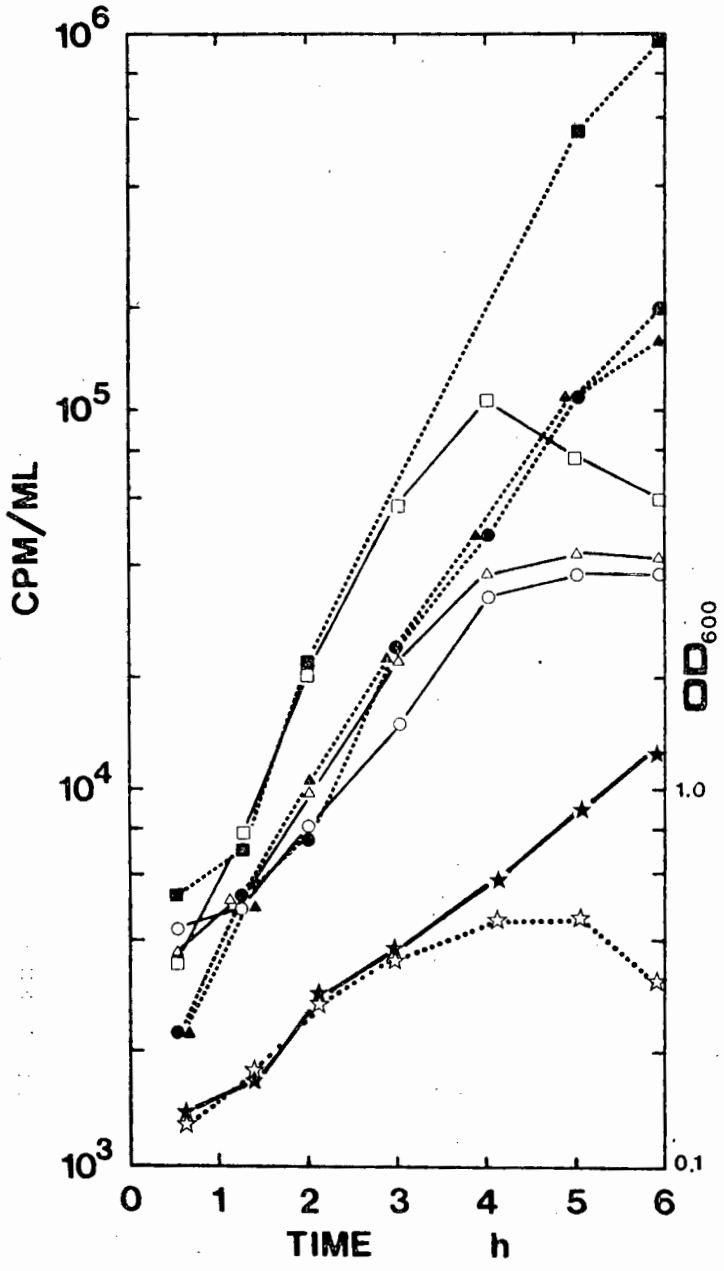


FIGURE 2. 7. A. Effect of bacteriocin on DNA,RNA and protein synthesis in B.fragilis BF-2 cultures. Cells were labelled with [^{14}C]thymidine with (○) and without (●) bacteriocin; [^{3}H]uracil with (□) and without (■) bacteriocin; and [^{35}S]methionine with (△) and without (▲) bacteriocin. Effect of bacteriocin on the optical density of a similar culture with (☆) and without (★) bacteriocin. The bacteriocin was added at 30min.

It was not possible to obtain a count of residual viable indicator bacteria during this experiment, possibly due to the presence of the bacteriocin in the samples. Attempts to wash the bacteria prior to plating out were frustrated by the susceptibility of the indicator strain to centrifugation , which resulted in variable and non-reproducible results. This poor recovery of B.fragilis following centrifugation may be due to aggregation of the bacteria or the death of some organisms.

2. 3. 4. REGULATION OF BACTERIOCIN PRODUCTION.

2. 3. 4. 1. INDUCTION OF BACTERIOCIN IN CULTURES.

Treatment of B.fragilis BF-1 with either Mitomycin C or UV irradiation resulted in no increased yield of bacteriocin.

2. 3. 4. 1. THE ABSENCE OF A PLASMID IN B.FRAGILIS BF-1

Plasmid DNA was not detected by any of the methods attempted.

2. 4. DISCUSSION

The low-molecular-weight bacteriocin, here designated J-BF-1, was produced by B.fragilis BF-1 in liquid culture and extracted either by suspending the cells in low-molarity buffer or by osmotic shocking and it is concluded that the bacteriocin activity was cell-bound.

The methods of bacteriocin extraction which proved successful were similar to those used for the extraction of the periplasmic protein fraction of E.coli (Neu & Hepple, 1965, Willis et al, 1974). Low molarity tris buffers have been shown to disrupt the integrity of the E.coli outer membrane, allowing the loss of periplasmic protein (Nikaido & Vaara, 1985) or allowing the utilization of substrates for which there is no uptake mechanism (Boye et al., 1981).

In E.coli alkaline phosphatase occurrs in the periplasmic protein fraction (Malamy & Horecker, 1961) and in Bacillus licheniformis it is extracellularly membrane-bound (Glynn et al., 1977). Alkaline phosphatase activity was detected in the crude bacteriocin extracts. The localization of alkaline phospatase in B.fragilis has not been reported.

Washing of B.fragilis BF-1 cells with 1M NaCl or 20% sucrose released relatively small quantities of bacteriocin compared to the tris buffer or distilled water extractions, so that it did not appear to be a mechanically detachable

material such as a capsule which contained the active bacteriocin.

Intracellular proteins were not observed in the buffer extracts of cells and there was no extensive cell lysis, suggesting the periplasmic location of the J-BF-1 bacteriocin of B.fragilis.

The mechanism of release of the colicins A, E-2 and E-3 has been suggested to be via the periplasmic space (Pugsley et al, 1984), but Cavard et al. (1984) have shown that there is no periplasmic accumulation of these colicins.

The properties of the inhibitor J-BF-1 are consistent with those of a bacteriocin since it is a bactericidal protein ith a narrow activity spectrum and is inactive against the roducer strain (Tagg et al., 1976).

The bacteriocin differs from that described by Mossie et al. (1979) which was produced extracellularly and inhibited RNA-polymerase activity in the sensitive strain immediately n addition. Purified J-BF-1 could not be shown to inhibit RNA-polymerase, in vitro or in vivo, nor did it cause gradation of radio-labelled RNA.

The differences between the two bactericins which have been eported from B.fragilis BF-1 are set out in Table 2. 6.

The size of J-BF-1 is similar to that of T1-1 from Bacteroides uniformis (Austin-Prather & Booth, 1984), and to that of the bacteriocin isolated from B.fragilis 1356 ayes et al., 1983), but their mode of action has not yet been reported.

TABLE 2.6

SUMMARY

of

PROPERTIES OF BACTERIOCINS K-BF-1 AND J-BF-1

PROPERTY	K-BF-1	J-BF-1
Sensitive indicator strain	B. fragilis Bf-2	B. fragilis BF-2
Presence in culture supernatant	about 1 600 AU/ml	200 -400 AU/ml
Low molarity cell extract	unknown	1 600 - 3 200 AU/ml
In vitro RNA-polymerase	Inhibited	Not inhibited
In vivo RNA synthesis	Inhibited	Not inhibited immediately
Effect on indicator strain	Bacteriostatic	lytic
Molecular size	16 000	7 000
Thermostability	Stable	Stable
Bacteriocin resistance related to rifampicin resistance	Related	Not related

The B.fragilis BF-1 strain which had been the source of the bacteriocin studied by Mossie et al (1979) no longer produced that bacteriocin and although attempts at reisolation of this strain were made at the start of this investigation, the bacteriocin could not be detected.

A recent report (Riley & Mee; 1985) of bacteriocins oduced from B.fragilis strains, identified three distinct types of bacteriocin, two of which corresponded to the bacteriocins K-BF-1 and J-BF-1 described here. Their bacteriocin A55 was cell bound and killed susceptible cells slowly. It affected the synthesis of protein, RNA and DNA simultaneously (similar to bacteriocin J-BF-1). Bacteriocin A49 was produced in the culture supernatant, had a rapid effect on indicator cells and was shown to inhibit RNA synthesis (similar to K-BF-1).

Although the K-BF-1 producer strain has been lost from the UCT culture collection, it is reassuring that another strain of B.fragilis, producing a RNA inhibiting bacteriocin, has been reported. This report only came to my attention after the work on the J-BF-1 bacteriocin had been completed and published (Southern et al.,1984).

The size of J-BF-1 is similar to that of T1-1 from Bacteroides uniformis (Austin-Prather & Booth, 1984), and to that of the bacteriocin isolated from B.fragilis 1356 (Hayes et al., 1983), but their mode of action has not yet been reported.

Attempts to induce greater bacteriocin production after treatment of the cells with UV light or Mitomycin C were unsuccessful. This lack of bacteriocin induction is common amongst bacterial genera unrelated to the Enterobacteriaceae (Hardy, 1975).

The effect of the bacteriocin on susceptible cells is an inhibition of DNA, RNA and protein synthesis accompanied by a decrease in optical density.

The mode of action of the bacteriocin J-BF-1 has not been identified, the long delay between the addition of the bacteriocin to susceptible cells and the inhibition of the synthesis of macromolecules suggests that the mode of action differs from that of most bacteriocins, which affect some aspect of cell metabolism relatively rapidly (within 10 min) (Konisky, 1982). Inhibition of macromolecular synthesis by J-BF-1 coincides with a decrease in culture turbidity, which suggests that the bacteriocin may be a lytic agent.

Pesticin A1122 acts as a lysozyme and has a protracted effect on susceptible cells before causing lysis and the inhibition of macromolecular synthesis (Ferber & Brubaker, 1979). Colicin M (Schaller et al., 1982), which also induces lysis of susceptible cells has been shown to be an inhibitor of murein biosynthesis.

Attempts to demonstrate rescue of indicator strain bacteria following bacteriocin treatment were unsuccessful due to the susceptibility of the bacteria to centrifugation and possible residual effects of the bacteriocin.

Pesticin A1122 acts as a lysozyme and has a protracted effect on susceptible cells before causing lysis and the inhibition of macromolecular synthesis (Ferber & Brubaker, 1979). Colicin M (Schaller et al., 1982), which also induces lysis of susceptible cells has been shown to be an inhibitor of murein biosynthesis.

Attempts to demonstrate rescue of indicator strain bacteria following bacteriocin treatment were unsuccessful due to the susceptibility of the bacteria to centrifugation and possible residual effects of the bacteriocin.

There was no evidence for the presence of an extra-chromosomal location for the genes coding for the bacteriocin but a plasmid was noted in the indicator strain and this was studied further in chapter 3.

CHAPTER 3.

CHARACTERIZATION OF THE CRYPTIC PLASMID FROM BACTEROIDES FRAGILIS BF-2 AND CONSTRUCTION OF A SHUTTLE VECTOR FOR B.FRAGILIS AND E.COLI

SUMMARY

The cryptic plasmid, pBFC1, from B.fragilis BF-2 was purified and a partial restriction map establised. Fragments were cloned into pBR325 in E.coli and the fragment from pBFTM10 which carries the clindamycin resistance gene was inserted into some of these pBFC1-pBR325 recombinant plasmids with the aim of developing a shuttle vector between the two bacterial species.

3. 1. INTRODUCTION.

3. 1. 1. Plasmids in Bacteroides.

The presence of plasmids in Bacteroides species is common (reviewed by Salyers, 1984). Many strains carry cryptic plasmids which have no distinguishable phenotype. Some of these plasmids appear to be related. Callihan et al. (1983) have shown that cryptic plasmids of less than 8 kb isolated from B.fragilis could be assigned to three homology classes on the basis of DNA hybridization.

Antibiotic resistance plasmids have also been reported in various species of Bacteroides (Magot et al, 1981; Privitera et al., 1979; Tally et al., 1979; Rogolsky et al., 1981).

Of particular interest are the transferrable plasmids pBF4 (Welch & Macrina, 1981) and pBFTM-10 (Tally et al., 1982), which both contain a clindamycin resistance gene (see Fig. 3. 4.). Analysis of these plasmids has shown that the clindamycin gene is homologous on both plasmids (Shimell et al., 1982; Guiney et al., 1984b; Robillard et al., 1985), and is contained within a compound transposon with direct DNA sequence repeats at each end; this stucture (Tn4400) has been shown to function in E.coli (Tally et al., 1982; Robillard et al., 1985). This clindamycin gene is widespread amongst Bacteroides strains and may be found on the bacterial chromosome as well as on the resistance plasmid (Marsh et al., 1983).

Transposon like structures have also been noted in the Bacteroides resistance plasmids studied by Magot et al, (1981). The transfer of these plasmids is believed to be by a form of conjugation (Welch et al., 1979), although this may differ from the conjugative mechanisms studied in the Enterobacteria. No evidence for the transfer of chromosomal genes by these plasmids has been noted. The transfer of tetracycline resistance however, may be by some non-plasmid mediated mechanism, which at present is uncharacterised (Privitera & Sebald, 1979; Macrina et al., 1981; Malamy & Tally, 1981; Mays et al., 1982; Smith et al., 1982).

A cryptic plasmid of B.fragilis BF-2 was found while searching for a possible plasmid in the BF-1 strain, which produces the bacteriocin, JBF-1, discussed in chapter 2. Although no evidence of a plasmid could be found in BF-1, the bacteriocin indicator strain was found to contain a small plasmid. This chapter describes the characterisation and cloning of that plasmid which has been designated pBF-C1.

3. 1. 2. SHUTTLE VECTOR PLASMIDS.

The development of plasmids which can replicate in more than one host species has been reported for a wide range of organisms. Bacillus - E.coli, Yeast - E.coli, mammalian cells - E.coli, B.fragilis - E.coli.

These are of use when analysing the effects of a gene from one species in another.

For a plasmid to be of use as a shuttle vector it must be capable of replication in both hosts and there must be a selectable marker. There should also be some second selectable marker for each strain which will allow the detection of in insertion.

The rationale employed in this study was that the small cryptic plasmid described in this chapter, can replicate at least in the BF-2 strain from which it was isolated, therefore it must contain an origin of replication. It has however, no detectable phenotype in B.fragilis or in E.coli. By linking fragments of the Bacteroides plasmid into pBR325, which contains E.coli antibiotic resistance genes for ampicillin, chloramphenicol and tetracycline, the hybrids would be selectable in E.coli. In order to include a marker which should be selectable in B.fragilis, the Cc^r (clindamycin) marker from the Bacteroides conjugal resistance plasmid pBFTM10 was ligated into the hybrid plasmids which had already been obtained.

At the time this work was commenced, no transformable shuttle vectors for B.fragilis had been reported and therefore the construction of such a vector was attempted.

In order to insert this plasmid into B.fragilis it was intended to use the polyethylene glycol facilitated transformation technique described by Smith in a personal communication, later published (1985a).

Once this had been achieved it was intended to further manipulate the plasmid as a functional shuttle vector (eg. Insert a cloning site cassette).

3. 2. MATERIALS AND METHODS.

3. 2. 1: BACTERIAL STRAINS:

Bacterial strains and plasmids were obtained from the culture collection of the University of Cape Town, Department of Microbiology. Those used in this study are listed in Table 3. 1.

The B.fragilis BF-2 strain has been described by Mossie et al. (1979) .

3. 2. 2: MEDIA FOR BACTERIAL GROWTH:

Bacteroides broth (Southern et al., 1984) was used for the growth of B.fragilis strains for the reasons outlined in Chapter 2. A complete description of the constituents and methods of preparation of media are given in appendix 2.

3. 2. 3: BUFFERS, SOLUTIONS AND ELECTROPHORESIS:

The formulations for the buffers used in this section are given in appendix 3. Electrophoretic techniques are described in appendix 4.

3. 2. 4: EXTRACTION AND PURIFICATION OF PLASMID DNA:

The method of Ish-Horowitz and Burke (1981) was used to extract plasmid DNA from E.coli. This was modified when working with B.fragilis. After the initial steps of cell lysis and precipitation of protein and chromosomal DNA, further purification by extraction with phenol/ chloroform/amyl alcohol (vols: 50/49/1) was necessary to remove Bacteroides proteins which were still present.

TABLE 3. 1.

BACTERIAL STRAINS AND PLASMIDS USED IN CHAPTER 3.

STRAIN	RELEVANT GENOTYPE	SOURCE or REFERENCE.
BACTEROIDES		
B.fragilis BF-2	pBFC1, bacteriocin indicator.	Mossie et al. 1979.
B.fragilis 4003	pBFTM10, clin^r.	Tally et al. 1979.
ESCHERICHIA		
E.coli K12 HB101	pro, leu, recA, amp^s, strep^r, hsdS$^-$(r$^-$, m$^-$),	Maniatis et al. 1982.
E.coli K12 CSR603	recA, uvrA, phr-1,	Sancar et al. 1979.
PLASMIDS		
pBFC1	Cryptic plasmid	This study.
pBFTM10	clin^r,	Tally et al. 1979.
pBR325	amp^r, tet^r, cml^r,	Bolivar, 1978.
pJS128a	tet^r, cml^r, (pBFC1-PstIa+b)	This study.
pJS128b	tet^r, cml^r, (pBFC1-PstIa)	This study.
pJS128c	tet^r, cml^r, (pBFC1-PstIb)	This study.
pJS128d	tet^r, cml^r, (pBFC1-PstIa+b)	This study.
pJS140z	tet^r, (pJS128a + pBFTM10-EcoRIb)	This study.
pJS140y	tet^r, (pJS128b + pBFTM10-EcoRIb)	This study.

Further purification of the plasmid DNA was undertaken by dye-bouyant centrifugation in CsCl containing ethidium bromide, followed by extraction with CsCl saturated iso-propanol and ethanol precipitation to remove these materials, and resuspension in TE buffer.

3. 2. 5: RESTRICTION MAP OF PLASMID pBFC1:

Restriction enzymes were obtained from Amersham International(UK), Bethesda Research Laboratories, Boehringer Mannheim and New England Nuclear. They were used according to the manufacturers directions. Standard techniques of single and double digests of plasmid DNA with a variety of restriction enzymes was used to establish the map of the plasmid (Maniatis et al., 1982). Restriction fragment sizes were estimated by comparison with molecular size standards of -DNA digested with the enzymes PstI, HinDIII and BstEII, which were electrophoresed in the same gel.

Electron micrographs of the plasmid DNA were prepared from samples spread onto carbon coated grids (Coetzee & Pretorius, 1979), stained with uranyl acetate, rotary shadowed with gold palladium and photographed using a Zeiss electron microscope (model E109). Measurements from the photographs were used to estimate the length of the plasmid molecule. Ten circular plasmid molecules were measured from a single preparation which included pDER401 (12.4kb) as an internal standard (Woods et al., 1986).

3. 2. 6: CURING OF B.FRAGILIS BF-2 OF THE pBFC1 PLASMID.

Attempts were made to cure the BF-2 strain of plasmid pBFC1 by growing it at 43-45°C, in the presence of acridine orange (16µg/mℓ), or in the presence of ethidium bromide (2µg/mℓ). These concentrations were shown to be the highest at which this strain of B.fragilis would grow and had proved successful in curing E.coli of the F'-lac plasmid. There have been no reports of plasmid curing in Bacteroides so that methods effective in E.coli were followed as a positve control. The strain was subcultured six times in Bacteroides broth under these conditions. Samples were streaked after each subculture and 10 colonies of each analysed for the presence of the pBFC1 plasmid.

3. 2. 7: CLONING OF FRAGMENTS OF pBFC1 INTO pBR325:

Purified pBFC1 and pBR325 DNA was digested to completion with PstI, phenol extracted to remove the enzymes, mixed in the proportions of 2 parts pBFC1 to 1 part pBR325 digest and ligated using T4 DNA ligase (Boehringer Mannheim). The ligated DNA was used to transform competent (Dagert & Erlich, 1979) E.coli HB101 cells and transformants were selected on Luria agar containing 150µg/ml chloramphenicol (cml). Recombinant plasmids were isolated by picking clones onto Luria agar containing 125µg/ml ampicillin (amp) and onto cml (150µg/mℓ). Clones which were cml^r and amp^s were screened for the presence of recombinant plasmids.

3. 2. 8: DETECTION OF PLASMID CODED PROTEINS:

The Prokaryotic DNA-directed translation kit (Amersham International UK) was used, according to the manufacturers directions, with purified DNA from pBFC1, pJS128a, pJS128b, pBR325, and pBFTM10. The [^{35}S]-methionine labelled translation products were analysed by SDS-PAGE and autoradiography.

3. 2. 9: DNA HYBRIDIZATION BETWEEN pBFC1 AND HYBRID CLONES:

Plasmid DNA from putative pBFC1-pBR325 recombinants was digested with PstI and the fragments separated by agarose electrophoresis in tris-acetate buffer. The DNA fragments were transferred to a nitrocellulose membrane (Hybond-N; Amersham, UK) according to Smith & Summers (1980). A [^{32}P]-labelled probe was prepared from purified pBFC1 DNA using a nick-translation kit (Amersham) according to the manufacturers directions. The membrane was treated according to the methods of Meinkoth & Wahl (1984) for prehybridization and hybridization. After washing, the membrane was placed on an X-ray film and exposed at -70°C for 72h.

3. 2. 10: CLONING OF THE pBFTM10 CLINDAMYCIN FRAGMENT:

Purified pBFTM10 DNA from B.fragilis 4003 was digested to completion with EcoR1 and mixed with similarly digested DNA from pJS128b and with pJS128c in a 1:1 ratio. After ligation (Maniatis et al., 1982) the DNA was used to transform competent E.coli HB101 cells and transformants were selected on Luria agar containing 20ug/ml tetracycline (tet).

Clones were screened for recombinants by picking onto tet and cml plates. Putative recombinants (tet^r, cml^s) were screened for the presence of hybrid plasmids.

3. 2. 11: TRANSFORMATION OF B.fragilis WITH PLASMID DNA:

The method of Smith (1985a) was used to transform B.fragilis BF-1 and BF-2 grown in medium containing 20mM $MgCl_2$ and made competent by treatment with polyethylene glycol (1000 MW). Purified plasmid DNA from pBFTM10, and from the recombinant plasmids (pJS140y and pJS140z) was used in the transformation attempts. Transformants were selected on brain heart infusion agar containing 5µg/mℓ clindamycin under anaerobic conditions.

3. 3. RESULTS

3. 3. 1. CHARACTERIZATION OF PLASMID pBFC1.

3. 3. 1. 1. EXTRACTION OF THE PLASMID pBFC1:

The B.fragilis BF-2 strain grew well in Bacteroides broth and a wet weight yield of bacterial cells of 10 - 15 g/l from an overnight culture was obtained.

The plasmid pBFC1 was readily seen on agarose electrophoresis of DNA extracts of B.fragilis BF-2, and using the modified Ish-Horowitz & Burke (1981) method of plasmid extraction, about 2mg of pBFC1 plasmid DNA could be obtained from a 2000ml overnight culture . This was approximately equivalent to the yield of plasmid obtained from E.coli (pBR325).

Extracts of pBFC1 which had not been purified by dye-bouyant centrifugation in CSC1 showed at least 3 bands on agarose electrophoresis, which correspond to the linear, nicked open circular and the supercoiled form of the plasmid (Fig. 3. 1). In addition a very much larger faint band was sometimes visible which may be due to the presence of concatemers of the plasmid or the presence of another very large plasmid (V.Abratt unpublished observations).

Using restriction enzymes which caused a single cut in the plasmid the size of the linear molecule could be estimated from agarose electrophoresis to be about 6 kilo-bases(kb). This was in agreement with estimates made from electron micrographs (Fig. 3. 2) of the plasmid which gave a size of 6.28kb (± 0.6kb).

3. 3. 1. 2. RESTRICTION MAP OF pBFC1:

Those restriction enzymes which did not to cut pBFC1 are AluI, ApaI, AvaI, BamHl, BclI, BglII, EcoRl, HinDIII, KpnI, PvuI, PvuII, SalI, SmaI, SstI, StuI, XbaI and XhoI. The restriction fragment lengths of pBFC1 which were obtained by digestion with various enzymes are set out in Table 3. 2.

The enzymes MluI and BglI caused a single cut in the plasmid and these were used to orientate the restriction sites of the other enzymes to obtain a map of the plasmid.

Because so many enzymes with 6-base recognition sites failed to cut the plasmid, some with 4-base recognition sites were used. This was not helpful as some cut the plasmid into many small fragments but Sau3Al was used to establish the map of the plasmid.

PstI digestion of pBFC1 gave rise to two fragments of approximately 3.2 and 2.8kb, labelled a and b respectively (Figure 3. 1).

The plasmid map obtained from these studies is shown in Figure 3. 3.

TABLE 3. 2.

Restriction fragment lengths obtained by digestion of pBFC1 with various restriction enzymes, estimated by agarose electrophoresis.

ENZYME NAME	RECOGNITION SEQUENCE	FRAGMENT LENGTH
BglI	5'-GCCNNNN*NGGC-3'	6.0
HaeIII	5'-GG*CC-3'	Many, less than 1kb.
HhaI	5'-GCG*C-3'	Many, less than 1kb.
MluI	5'-A*CGCGT-3'	6.0
PstI	5'-CTGCA*G-3'	3.2(a), 2.8(b).
RsaI	5'-GT*AC-3'	1.5, 0.94, 0.48 and many small fragments
Sau3AI	5'-*GATC-3'	2.4, 2.0, 0.95, 0.65
TaqI	5'-T*CGA-3'	Many, less than 1kb.

*=cleavage point.

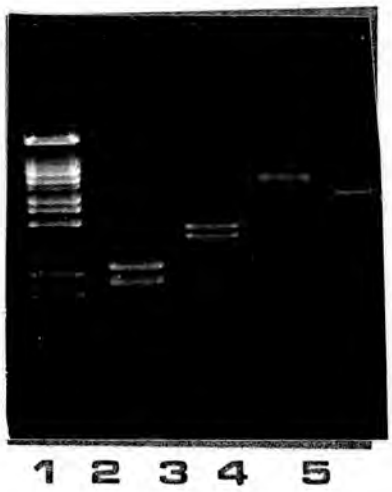


Figure 3. 1.: Agarose electrophoresis of a restriction digest of pBFC1. Approximately 3ug of DNA from restriction digests were electrophoresed in a 0.7% Biorad agarose in tris-borate buffer and stained with ethidium bromide (fragment sizes). Lane 1; λ-*Bst*EII markers (48.5-0.7kb), 2; pBFC1-*Sau*3AI (2.4, 2.0, 0.95, 0.65kb), 3; pBFC1-*Pst*I (3.2, 2.8kb), 4; pBFC1-*Mlu*I (6.0kb), 5; pBFC1 undigested.

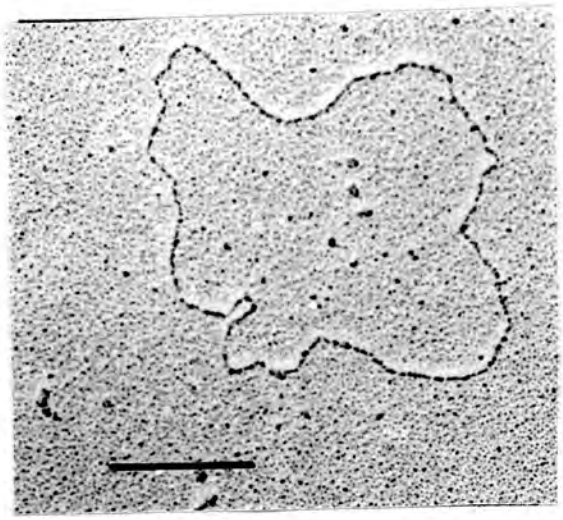


Figure 3. 2. Electron micrograph of plasmid pBFC1 prepared as described in the text. The bar represents 0.5kb.

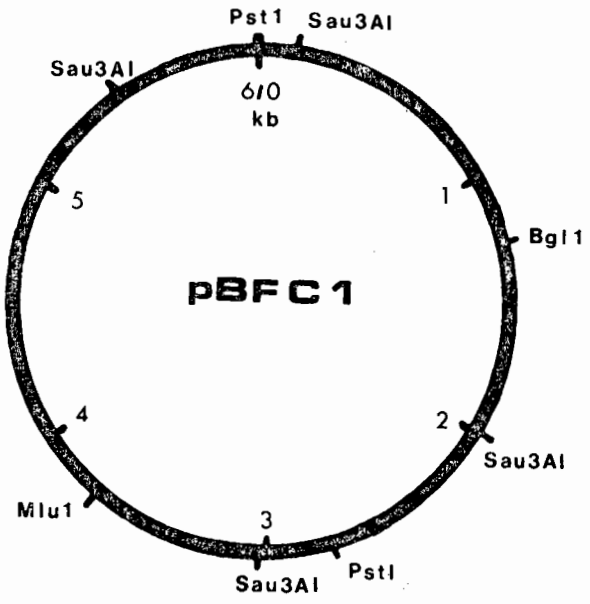


Figure 3. 3. Restriction map of the plasmid pBFC1 from B.fragilis, based on fragment sizes estimated from agarose electrophoresis.

3. 3. 2. CLONING OF FRAGMENTS OF pBFC1 IN pBR325

3. 3. 2. 1.

Fragments of the plasmid pBFC1 were cloned into the vector pBR325 in E.coli HB101. pBR325 has three selectable antibiotic markers, ampicillin (amp), chloramphenicol (cml) and tetracycline (tet). Each of these has restriction sites, unique for pBR325, which allow the insertional inactivation of the antibiotic marker. The only known unique restriction sites in pBFC1 were those for BglI and MluI and as pBR325 does not have a suitable site to allow insertion of fragments bounded by these ends, it was decided to clone the two PstI fragments from pBFC1.

A total of 51 cml^r, amp^s clones were selected for screening. Clones containing each of the fragments were found and a clone which contained both fragments. From restriction mapping experiments this latter clone appears to have the pBFC1 PstI fragments in the same orientation as in the native plasmid (Fig.3. 4.).

The recombinant plasmids are designated as follows:

pJS128a	pBR325 + pBFC1(PstIa + PstIb)
pJS128b	pBR325 + pBFC1(PstIb)
pJS128c	pBR325 + pBFC1(PstIa)

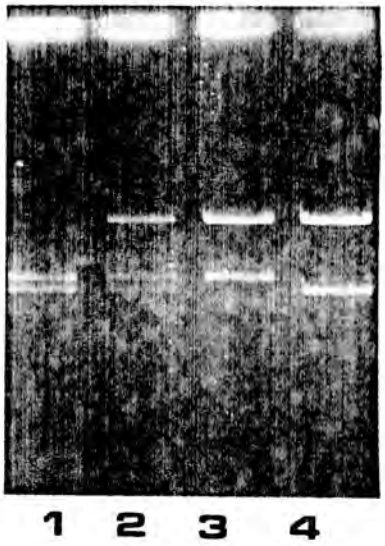


FIGURE 3. 4: Agarose electrophoresis of PstI restriction digests of pBFC1-pBR325 hybrid plasmids. Samples of approximately 5ug of DNA from restriction digests were electrophoresed in a 1.0% Biorad agarose gel and stained with ethidium bromide (fragment sizes). Lane 1; pBFC1-PstI (a: 3.2, b: 2.8kb),
2; pJS128a-PstI (pBR325: 5.4, a: 3.2, b: 2.8kb),
3; pJS128b-PstI (pBR325: 5.4, a: 3.2kb),
4; pJS128c-PstI (pBR325: 5.4, b: 2.8kb).

3. 3. 2. 2. DNA HYBRIDIZATION:

DNA hybridization studies showed that the fragments of DNA which were inserted into pBR325 were derived from the pBFC1 plasmid of B.fragilis BF-2 (Fig.3. 5.).

The autoradiograph shows a clear hybridization signal between the putative pBFC1 fragments inserted into pBR325 and the [^{32}P]-pBFC1 probe. There was no detectable homology between pBFC1 and pBR325.

3. 3. 2. 3. EXAMINATION FOR PLASMID CODED PROTEINS:

The in-vitro transcription/translation kit resulted in the production of several polypeptides from pBFC1 DNA. They were of the following apparent Mr: 90000(faint), 65000, 50000, 48000, 47000, 46000, 45000, 39000, 32000, 23000. The translation products of the vector plasmid pBR325 were visible, Mr; 34000(tet), 28000(Amp), 22500(cml) (Covarrubias et al. 1981).

The hybrid plasmids each produced some of the polypeptides encoded by the undigested pBFC1 plasmid. pJS128b produced the following polypeptides of apparently similar size to those of the pBFC1 plasmid; Mr 90000, 65000(faint), 50000, 48000, 47000, 46000, 45000, 32000, 23000. pJS128c produced the following polypeptide; 39000. See Fig. 3. 6.

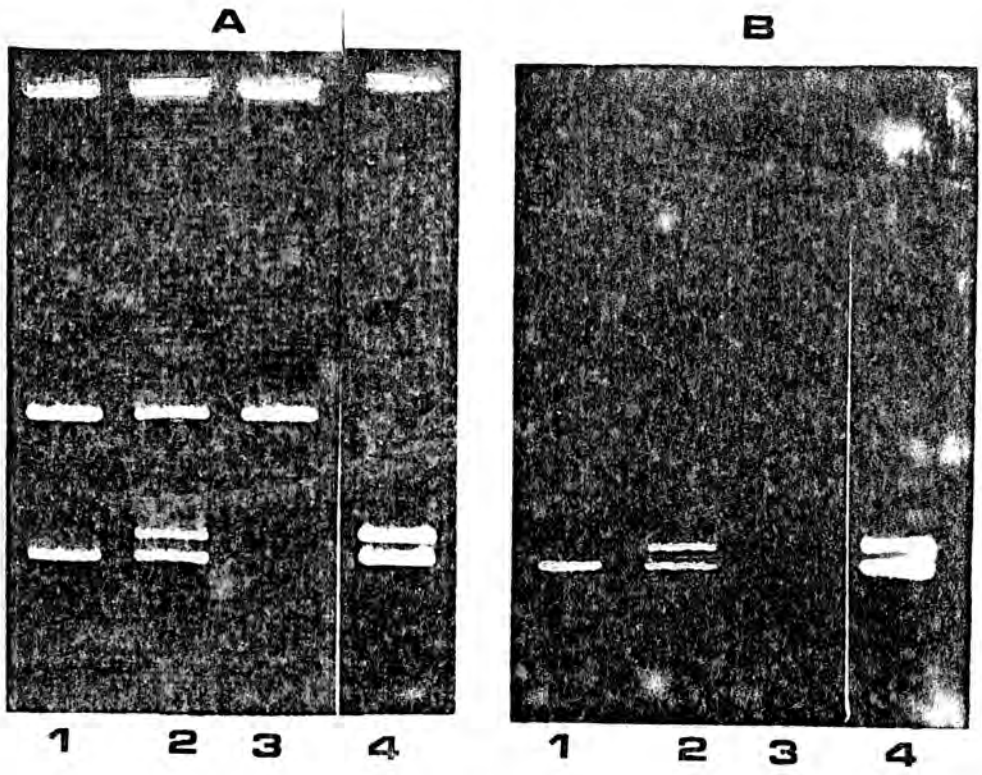


FIGURE 3. 5. Hybridization of [^{32}P]-pBFC1 and plasmid DNA. A: Ethidium bromide stained agarose electrophoresis of PstI restriction digests of plasmid DNA, Lane 1,pJS128b (pBR325-pBFC1/b hybrid), 2, pJS128a (pBR325-pBFC1/a+b hybrid), 3, pBR325, 4, pBFC1.

B: Autoradiograph of a Southern blot of the gel in A.

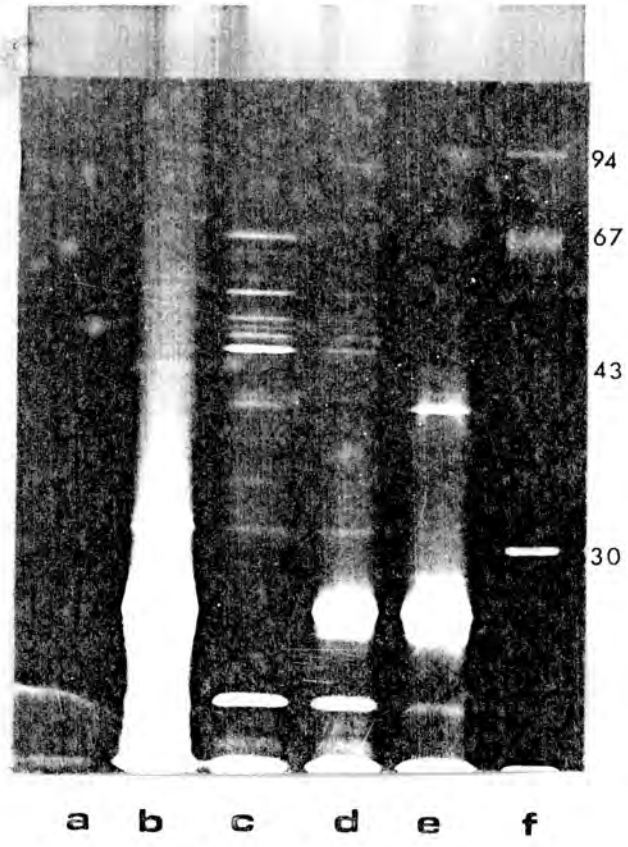


FIG. 3. 6. SDS-PAGE analysis of labelled polypeptides produced from the in-vitro transcription/translation kit syntheses, using equal quantities of the following DNA samples as template:

a: No DNA control. b: pBR325. c: pBFC1. d: pJS128b. e: pJS128c. f: MW: Mr markers X 10^3.

3. 3. 2. 4. CURING B.FRAGILIS BF-2 OF PLASMID pBFC1:

No isolates of B.fragilis BF-2 treated with either growth at high temperature, in the presence of acridine orange or with ethidium bromide were found to be cured of the plasmid pBFC1.

3. 3. 3:

3. 3. 3. 1: PREPARATION OF pBFC1/pBR325/pBFTM10 HYBRIDS:

The restriction enzyme EcoRI cuts the clindamycin resistance plasmid pBFTM10 into three fragments. The middle sized, b', fragment (4.4 kb) contains the clindamycin resistance gene (Guiney et al., 1984).

Unless the pBFC1 origin of replication had been damaged by the PstI digestion used in cloning fragments of the plasmid into pBR325, one of the two recombinant plasmids obtained must contain an origin of replication, functional in B.fragilis.

EcoRI digests of pBFTM10, pJS128b, and pJS128c were mixed, ligated and transformed into competent E.coli HB101 cells. This gave rise to several hundred tet^r transformants.

These in turn gave rise to 44 and 18 clones respectively which were amp^s, cml^s and tet^r.

These clones were screened by plasmid analysis and two recombinant plasmids were found:

pJS140z, which incorporated into pBR325 the larger (a) PstI fragment of pBFC1 together with the b (Clin) fragment of pBFTM10.

pJS140y which contained the smaller (b) PstI fragment of pBFC1 and the b fragment of pBFTM10 (Figure 3. 7.).
This is shown diagrammatically in Fig.3. 8.

As expected, none of the hybrid clones were resistant to clindamycin (20ug/mℓ). Although the pBFTM10 fragment also carries a cryptic tet^r marker expressed by E.coli during aerobic growth (Guiney et al., 1984c), this was not detected because the pBR325 is already resistant to tet.

No $clin^r$ clones were obtained after attempts to transform calcium shocked (Davis et al., 1980) or polyethylene glycol treated (Smith, 1985a) B.fragilis BF-2 and B.fragilis BF-1 strains with purifed DNA from plasmids pBFTM10, pJS140y and pJS140z.

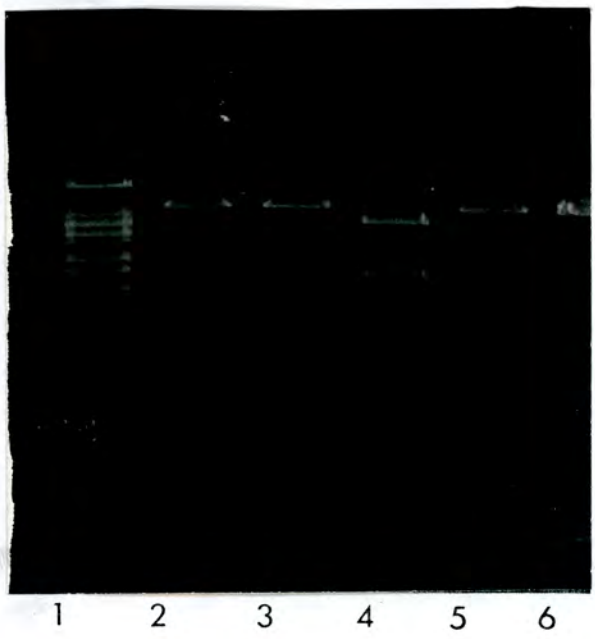


Figure 3. 7. pBR325-pBFC1-pBFTM10 hybrid plasmids. Samples of approximately 5ug of DNA from restriction digests were electrophoresed in 0.8% Biorad agarose in tris-borate buffer and stained with ethidium bromide (fragment sizes). Lane 1; λ-BstEII markers (48.5 - 0.7kb), 2; pJS128b-EcoRI (8.6kb), 3; pJS140z-EcoRI (undigested, 8.6, 4.4kb), 4; pBFTM10-EcoRI (7.35, 4.4, 2.5kb), 5; pJS140y-EcoRI (8.2, 4.4kb), 6; pJS128c-EcoRI (8.2kb).

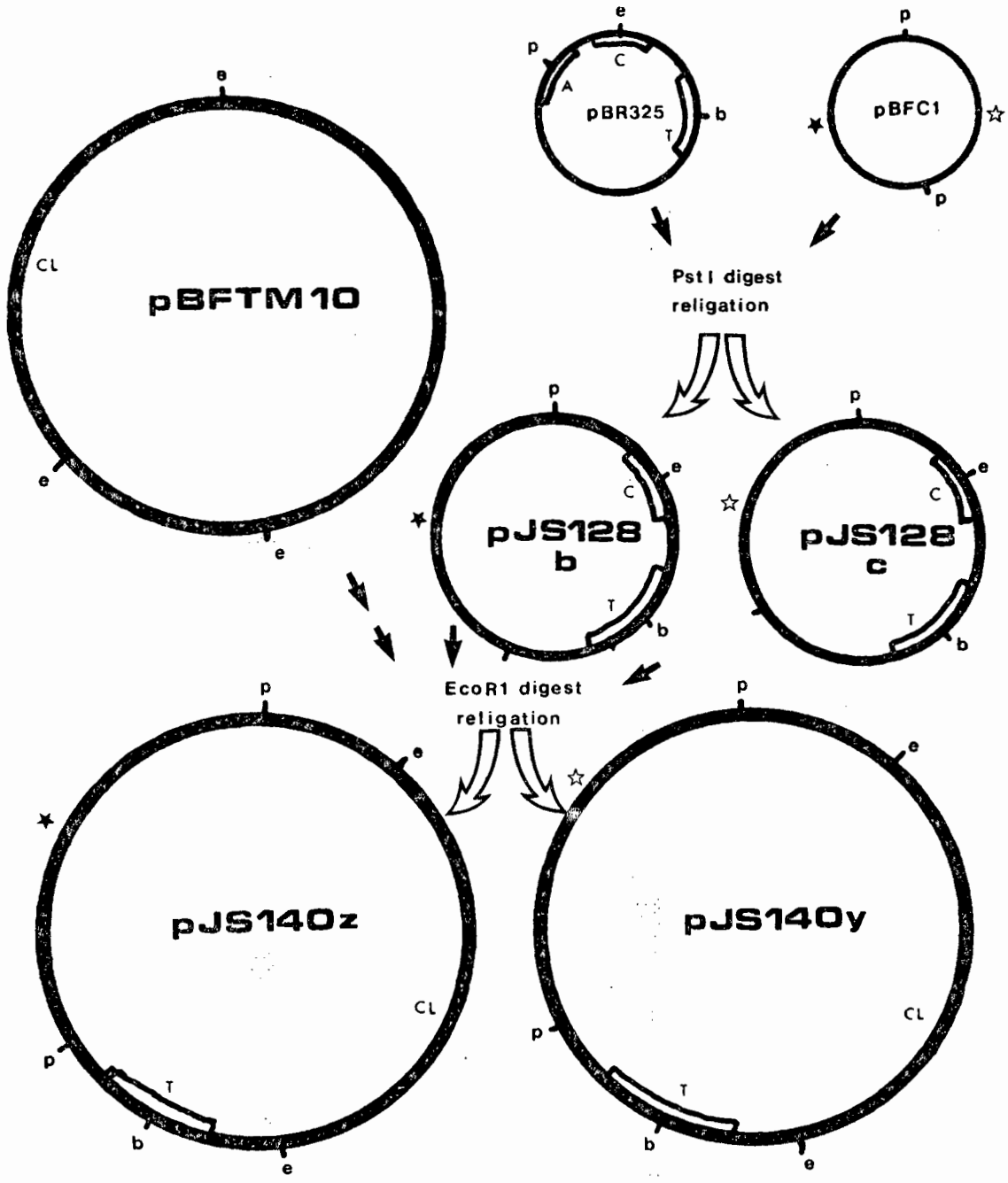


FIGURE 3. 8. Diagram showing the derivation of plasmids pJS140y and pJS140z. Restriction sites; p=PstI, e=EcoRI, b=BamHI. Antibiotic resistance genes; A=ampicillin, C=chloramphenicol, T=tetracycline, CL=clindamycin (in B.fragilis).

SECTION 6: DISCUSSION

A cryptic plasmid (pBFC1) from the B.fragilis BF-2 strain was extracted and purified and a restriction map established of the molecule. It has not been possible to acquire sufficient published data to make a meaningful comparison of the restriction map of this plasmid and other small cryptic Bacteroides plasmids. The plasmid pBFC1, showed no restriction map homology with larger antibiotic resistance plasmids (eg. pBFTM10 and pBF4)

There was no detectable phenotype for the plasmid, and it has been suggested (Salyers, 1984) that these cryptic Bacteroides plasmids may be little more than parasites, which encode only the functions needed for their own replication. Attempts to cure the strain of the plasmid were not successful, but with no marker, cured strains may have been missed.

The restriction mapping of the plasmid was made more difficult because of the lack of restriction sites for enzymes which have six-base recognition sites. It did not appear that this was due to modification of the plasmid DNA by the host, B.fragilis BF-2, as the fragments which were cloned into the restriction-modification minus strain E.coli HB101 were found not to differ in their susceptibility to these restriction enzymes. No restriction-modification system in B.fragilis has been described.

The PstI fragments of pBFC1 were successfully cloned into pBR325. Unless inactivated by the PstI digestion and religation, one of these clones should contain the origin of replication for B.fragilis. The polypeptides produced by pBFC1 in the in-vitro system were mostly produced by one or other of the cloned fragments. Whether any of these represent functional proteins is not known. The presence of these in-vitro translation products indicates that E.coli transcription/translation enzymes recognize promoter sequences on the Bacteroides plasmid DNA, and translation start, and termination signals, whether these are meaningful signals in B.fragilis is not known, but these results encouraged further experimentation which detected functional B.fagilis gene expression in E.coli.

As the pBR325 antibiotic resistance genes are not expressed in Bacteroides (Guiney et al., 1984c), the fragment of DNA from the pBFTM10 $clin^r$ plasmid which is believed to carry the $clin^r$ determinant was inserted into the cml gene of pBR325 carried on the pJS128 series of plasmids.
These hybrid plasmids did not express $clin^r$ in the E.coli HB101 host. It has not been possible to transform these plasmids into the available strains of B.fragilis . Smith, (1985a, 1985b) reports successful transformation of plasmid DNA into B.fragilis strain 638, but the methods have not proved successful with the available B.fragilis strains. This was confirmed by Smith (1985a) who reported the failure of his transformation procedure with strains of B.fragilis other than strain 638.

He did mention that he had had limited success using another vector with other strains but these were not specified. Strain 638 was not available at the time these experiments were being carried out, and the development of the shuttle vectors by Smith (1985 b) made the continuation of this study redundant. Emphasis was therefore shifted to the study of B.fragilis glutamine synthetase.

The pJS140 plasmid series are constructed so that at least one should both replicate and be selectable in B.fragilis and E.coli. This putative shuttle plasmid contains the pBR325 origin of replication (functional in E.coli), and the origin of replication from PBFC1 (functional in B.fragilis), as well as an antibiotic marker selectable in E.coli (tet^r) and another which may be selectable in B.fragilis ($clin^r$). No mobilization studies with broad host range plasmid systems have been attempted with either pBFC1 or its derivatives. The reason for this is the unsuitability of the antibiotic resistance markers (tet) on the broad host range mobilizing plasmids available.

If the difficulties with transformation of B.fragilis could have been overcome, this putative plasmid vector may have been of value in the analysis of Bacteroides genes, and in analysing some of the physiological characteristics of anaerobic organisms.

This investigation had not resolved the problem of whether Bacteroides genes could be expressed in E.coli, and it was decided to prepare a genomic library of B.fragilis BF-1 chromosomal DNA and screen this for the expression of Bacteroides genes.

CHAPTER 4

THE CONSTRUCTION OF A BACTEROIDES FRAGILIS BF-1 GENETIC LIBRARY IN pEcoR251

SUMMARY

A genetic library of DNA extracted from B.fragilis BF-1 was established in the positive selection vector pEcoR251. This library was screened for expression of Bacteroides genes by the complementation of E.coli mutant strains. Complementation of auxotrophic markers was observed, as was resistance to mitomycin C. A clone was isolated which complemented the glutamine synthetase (glnA) deficiency in an E.coli deletion strain.

INTRODUCTION

The study of microbial molecular genetics has been greatly assisted by the establisment of libraries or banks consisting of randomly sized fragments of chromosomal DNA from the organism under investigation which have been cloned into a suitable vector plasmid.

Such libraries have been established for many organisms, using a variety of vector systems. The use of *in vitro* assembled recombinant phages (cosmids) are favoured by some workers as the size of the insert can be particularly large (± 40kb) and selection of a particular gene can be made from a relatively small number of recombinants (Hohn & Collins, 1980, Ish-Horowitz & Burke, 1981).

Other methods include the insertion of the DNA fragment into a position on the vector where it will be transcribed from a promoter carried by the vector, this is particularly useful if the inserted DNA is not likely to be expressed in the *E.coli* host.

One of the problems encountered in these investigations was the separation of recombinant plasmids from the background of reassembled vectors. Several elegant systems are available, for example only recombinant cosmids are viable and able to package in the *in vitro* system of Hohn & Collins (1980).

The pEcoR251 plasmid was derived from the pCL plasmids described by Zabeau & Stanley (1982) and consists of the *E.coli* *Eco*R1 gene under the control of the λ-rightward

promoter, the ampicillin resistance (amp) gene and the pBR322 origin of replication. The EcoR1 gene product, expressed at high levels by the λ-promoter on pEcoR251, is lethal unless insertionally inactivated or regulated by plasmid pC_{I857}, which contains a temperature sensitive λ-repressor gene (Remaut et al., 1983). The EcoR1 gene has a single Bgl1I restriction enzyme site which can be used for the insertion of DNA digested with Bgl1I, BamH1, or Sau3A1.

There has been some doubt regarding the possibility of expression of Bacteroides genes in other bacteria following a report by Guiney et al. (1984c) which indicated that the ampicillin resistance gene from E.coli was not expressed in B.fragilis and that the clindamycin resistance gene from the B.fragilis pBFTM10 plasmid was not expressed in E.coli. However the cloning and expression in E.coli of the fimbrial sub-unit protein, pilin protein, cellulase, and chondroitin lyase genes from Bacteroides nodosus, Bacteroides succinogenes, and B.thetaiotaomicron have been reported (Anderson et al., 1984, Crosby & Collier, 1984, Elleman & Hoyne, 1984, Guthrie et al., 1985).

The utilization of proteins and amino-acids by Bacteroides strains has been reviewed by Salyers (1984), who concludes that although most strains cannot use amino-acids as a sole

carbon source, the uptake and incorporation of at least some is proven. In chapter 2 the uptake and incorporation of methionine was successfully used as a marker of protein synthesis. The growth of Bacteroides on minimal media without supplemental amino-acids (Varel & Bryant 1974) shows that they must have functional pathways for the synthesis for the required amino-acids, although these pathways may not be the same as those of E.coli (Alison et al., 1984). Thus the presence of clones which complement amino-acid deficiencies in E.coli were sought in the Bacteroides gene library.

As discussed in Chapter 1, B.fragilis has been shown to have mechanisms which capable of repairing DNA damaged by UV irradiation (Schumann et al., 1982, Slade et al., 1983 and 1984) or mitomycin C. Both agents cause DNA damage by the dimerization of adjacent thymine residues on the DNA (Friedberg, 1985), and the repair systems operative in E.coli excise these bases and replace them with undamaged thymine. The B.fragilis gene library was screened for a clone which could confer increased resistance to mitomycin C in a repair deficient strain of E.coli.

The secretion of proteolytic enzymes and extracellular DNase by strains of B.fragilis has been reported (reviewed by Salyers, 1984) and the presence of a functional protease or DNase secreting clone was sought in the gene library.

Glutamine synthetase(GS) is an important enzyme in the pathways of nitrogen metabolism for many bacteria and as it's presence in B.fragilis was anticipated, the gene library prepared from the B.fragilis BF-1 strain was screened for the expression of this enzyme.

4. 2. MATERIALS AND METHODS

4. 2. 1: BACTERIAL STRAINS AND PLASMIDS.

A list of the bacterial strains and plasmids used in this study are given in Appendix 1. The B.fragilis BF-1 strain has been described by Mossie et al. (1979). The plasmid pEcoR251 was a gift from M.M.Zabeau of Plant Genetic Systems, Ghent, Belgium.

4. 2. 2: BACTERIAL GROWTH MEDIA AND CONDITIONS.

B.fragilis BF-1 was grown in bacteroides broth or the minimal medium of Varel & Bryant (1974). E.coli strains were grown in Luria broth or in CSH minimal medium, supplemented as described in the text (Davis et al., 1980). The formulations and methods of preparation of all the media are given in Appendix 2.

4. 2. 3: BUFFERS, SOLUTIONS AND ELECTROPHORESIS.

The formulations of buffers and solutions and the methods of agarose and polyacrylamide gel electrophoresis are set out in Appendices 3 and 4.

4. 2. 4: PREPARATION OF DNA.

Plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowitz & Burke (1981) and the B.fragilis chromosomal DNA was prepared as follows:

Bacterial cells collected from 100ml of a late log phase

culture (about 10^9 cells/mℓ) were suspended in 5mℓ of 0.01M tris-HCl buffer (pH 7.8) containing 10mM EDTA and 8.0mM glucose. S.griseus protease (Pronase E, Sigma) (10mg) and 100mg of sodium dodecyl sulphate (SDS) was added and gently mixed to dissolve. The resulting cell lysate was extracted with a phenol/chloroform/amyl alcohol (vols:50/49/1) (Marmur, 1961) mixture to remove proteins and lipids until the supernatant was clear. After precipitation with an equal volume of iso-propanol, the pellet was redissolved in 5ml of TE buffer.

4. 2. 5: CONSTRUCTION OF A B.FRAGILIS GENE LIBRARY:

The purified chromosomal DNA was digested with sufficient Sau3AI restriction endonuclease (Boehringer-Mannheim) to produce a spectrum of DNA fragment sizes and fractionated on a sucrose density gradient. DNA was precipitated from those fractions which were shown by agarose electrophoresis to contain fragments of 3 - 10 kilobases. The purified plasmid pEcoR251 DNA was digested to completion with BglII, precipitated with ethanol and redissolved in TE buffer.
After ligation (Davis et al., 1980), the DNA was used to transform competent (Dagert & Erlich 1979) E.coli HB101 cells and the transformants were selected on Luria agar containing amp (125ug/mℓ). E.coli HB101 amp^r colonies were pooled and recombinant plasmid pEcoR251 DNA extracted and stored at -70°C.

4. 2. 6: SCREENING FOR AUXOTROPHIC MARKERS:

E.coli HB101 is deficient in the genes for the production of the amino-acids proline (pro) and leucine (leu). Approximately 10ug of the pooled DNA was transformed into 10^9 competent cells of this strain and the transformants were selected directly on CSH minimal medium containing amp (125ug/mℓ) and either proline (1mg/mℓ) or leucine (1mg/mℓ). Colonies which appeared were screened for the presence of recombinant pEcoR251 plasmids and for their ability to grow on the appropriate amino-acid supplement.

4. 2. 7: SCREENING FOR MITOMYCIN C RESISTANCE:

The minimum inhibitory concentration of mitomycin C for E.coli HB101 recA$^-$ was determined by dilution in Luria agar plates at concentrations between 0.05 amd 0.15ug/mℓ. A concentration of 0.25ug/mℓ mitomycin C and 125ug/mℓ amp was used to select transformants after transformation of approximately 10^9 competent cells with 10ug of the pooled B.fragilis gene library. Apparent resistant clones were checked for the presence of recombinant pEcoR251 plasmid and for evidence of increased resistance to UV irradiation as well as hybridization to Southern blots of digests of chromosomal DNA.

4. 2. 8: SCREENING FOR PROTEASE PRODUCTION:

Eight ug of the pooled B.fragilis gene library was transformed into 10^9 competent cells of E.coli HB101 which does not produce an extracellular protease.

Transformants were plated onto Luria agar containing amp(125ug/mℓ) and dried skim milk powder (5g/1) and incubated for 24h at 37°C followed by 72h at 20 - 25°C and the colonies were examined for a clear zone surrounding a protease producing clone.

4. 2. 9: SCREENING FOR DNASE PRODUCTION:

Eight ug of the pooled B.fragilis gene library was transformed into 10^9 competent cells of E.coli HB101 which does not produce an extracellular DNase. Transformants were plated onto DN-ASE agar (Oxoid, UK) containing amp (125ug/mℓ) and toluidine blue (0.01mg/mℓ) and incubated for 24h at 37°C followed by 72h at 20 - 25°C. The colonies surrounded by a clear zone were indicative of a DNase producing clone.

4. 2. 10: SCREENING FOR GLUTAMINE SYNTHETASE PRODUCTION:

Ten ug of the pooled B.fragilis gene library was transformed into 10^9 competent cells of the E.coli YMC-11 glnA, ntrB, ntrC deletion mutant strain and GS transformants were selected on minimal medium containing $(NH_4)_2SO_4$ as the sole source of nitrogen.

4. 3. RESULTS

4. 3. 1: PREPARATION OF A B.FRAGILIS BF-1 GENOMIC LIBRARY:

Following transformation of E.coli HB101 with ligated pEcoR251 and B.fragilis chromosomal DNA, a total of more than 10000 amp^r colonies was obtained.

These colonies were scraped from the plates into four pools and the plasmid DNA extracted and stored at -70°C.

A DNA concentration of approximately 1mg/mℓ was obtained.

Agarose electrophoresis indicated that a wide spectrum of insert sizes had been obtained with the majority in the region of 8 to 20 kb (Figure 4. 1). A transformation frequency using this DNA with competent E.coli HB101 cells, of approximately 10^5 amp^r clones per ug DNA was obtained.

Because pEcoR251 has two PstI restriction sites which cut the plasmid into two fragments of approximately 1.9kb(a) and 1.2kb(b); a PstI digest of a putative recombinant plasmid followed by agarose electrophoresis allows a quick estimate of the size of the DNA insertion, as one band(1,9kb) on the gel will be derived from the vector, while the others will consist of the other part of the vector (1.2kb) plus the inserted DNA fragment (Figure 4. 2).

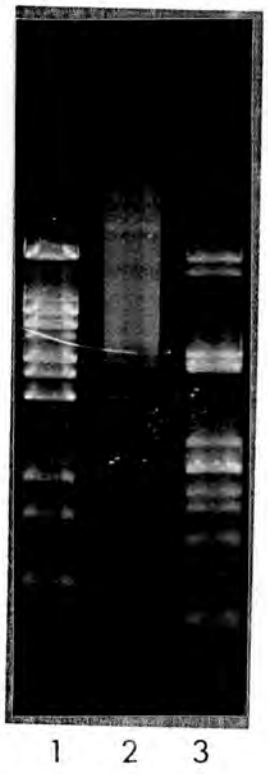


Figure 4. 1. Indication of the size of DNA inserts obtained in the gene library. Approximately 5ug of DNA from the B.fragilis gene library was electrophoresed on a 0.8% Biorad agarose gel and stained with ethidium bromide. Lane 1; λ-BstEII markers (48.5-1.2kb), 2; purified sample of library DNA, 3; λ-PstI markers.

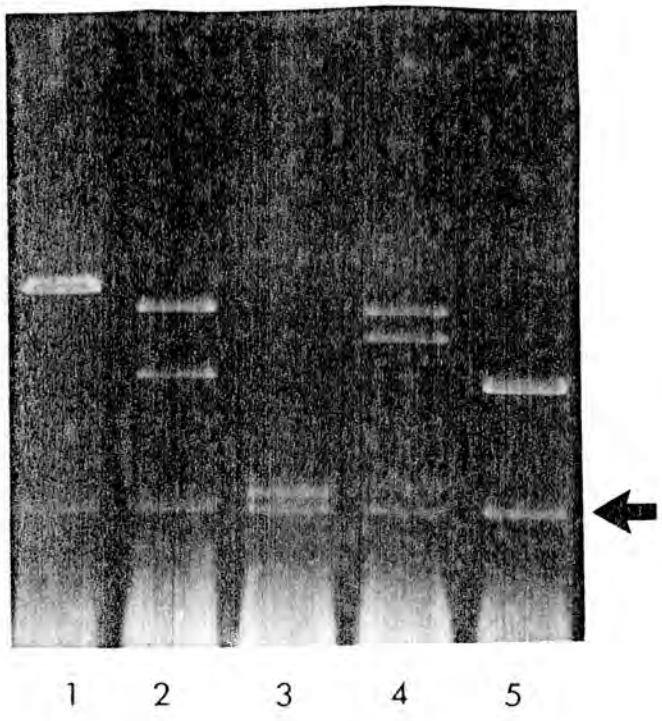


Figure 4. 2.

Analysis of recombinant plasmids selected at random from the gene library. Approximately 5ug of a PstI restriction digest of plasmid clones from the B.fragilis gene library was electrophoresed on a 0.8% Biorad agarose gel and stained with ethidium bromide. The arrow indicates the pEcoR251 PstIa fragment (1.8kb).

4. 3. 2: ISOLATION OF AUXOTROPHIC MARKERS:

Four clones of HB101 were obtained following transformation with the pooled B.fragilis gene library which were amp^r and leu^+. These clones contained a recombinant plasmid but were not analysed further (Fig 4. 3.).

Similarly a single recombinant clone which was amp^r pro^+ was obtained.

4. 3. 3: EXPRESSION OF MITOMYCIN C RESISTANCE:

A small colony was detected on a plate containing 0.25ug/ml of mitomycin C. This colony was subcultured and proved to contain a recombinant pEcoR251 plasmid with a 5kb insertion (Fig 4. 4). However on retransformation, the expression of mitomycin C resistance was very difficult to demonstrate consistently. The minimum inhibitory concentration appeared to be between 0.25 and 0.2 ug/mℓ, which was very close to that of the sensitive host strain. There was no evidence of increased resistance of the clone to the effects of UV-irradiation. DNA hybridization of this plasmid to digests of chromosomal B.fragilis DNA showed that the insert had most probably been derived from this strain. Because of the poor growth of the clone on media which contained sub-inhibitory concentrations of mitomycin C, it was not possible to maintain this clone under selective pressure and investigations as to its nature were discontinued.

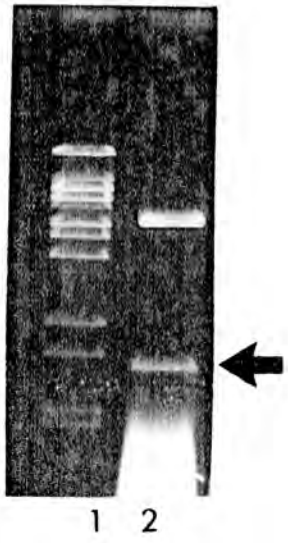


Figure 4. 3.

The leu$^+$ plasmid from the B.fragilis gene library. Approximately 5ug of DNA from a PstI restriction digest of the plasmid clone which complemented the Leu deficiency of E.coli HB101 was electrophoresed on a 0.8% Biorad agarose gel and stained with ethidium bromide. Lane 1; λ-BstEII markers (48.5 - 0.7kb), 2; Leu plasmid digest. The arrow indicates the pEcoR251 PstIa fragment (1.8kb).

4. 3. 4: PROTEASE AND DNASE PRODUCTION:

No clones were isolated which showed evidence of the production of either an extracellular protease or an extracellular DNase.

4. 3. 5: PRODUCTION OF GLUTAMINE SYNTHETASE:

Four colonies grew on the minimal medium amp plates following transformation of the E.coli YMC-11 glnA deletion strain with the gene library prepared from B.fragilis BF-1. Plasmid analysis showed that they all contained a recombinant pEcoR251 plasmid with approximately the same sized insert.

One of these was studied further (chapter 5).

Retransformation of E.coli YMC-11 glnA and E.coli ET8501 glnA showed that the GlnA phenotype was carried by the plasmid, as approximately equal numbers of transformants were obtained on Luria agar containing amp as on the CSH minimal medium plates.

SECTION 5: DISCUSSION

A genetic library was prepared from B.fragilis BF-1 in the vector pEcoR251 and the success of this manipulation was concluded from the expression of functions by plasmids derived from that library.

The number of clones which were combined in the gene library and their approximate average size would suggest that at least a major portion of the B.fragilis BF-1 genome was represented. This strain could not be shown to carry a multi-copy plasmid (see chapter 2), so that the presence of an unbalanced representation of plasmid genes was not likely.

Any expression of inserted DNA may be from the λ-rightward promoter carried by the pEcoR251 vector or it may be from promoter sequences carried by the insert, which are recognized by the E.coli host transcriptional mechanisms. These promoters need not necessarily be the same as those recognized by B.fragilis.

The expression of amino-acid markers in E.coli indicated that certain synthetic pathways may be analogous between E.coli and B.fragilis. The whole synthetic pathway may have been transferred by the insertion as Alison et al. (1984) have demonstrated the function of alternative leucine biosynthetic pathways in Bacteroides ruminicola and B.fragilis.

The clone which appeared to express resistance to mitomycin C was not suitable for analysis, the difference between the minimum inhibitory concentration of the drug for the host organism and the level of resistance conferred by the recombinant plasmid was so small that it was frequently difficult to differentiate between the clone and the control. As there was no evidence of UV repair it was concluded that the clone may have had some alteration to an uptake mechanism or alternatively was able to reduce the mitomycin C before it could damage the host chromosome (mitomycin C is rapidly inactivated by reducing agents).

This clone was not investigated further in this study, as rescreening of this library by researchers at the University of Cape Town using the selective agent methylmethane sulphonate have isolated a stable clone which appears to confer resistance to DNA damaging agents on its E.coli host (H.Slade personal communication). This clone also confers a measure of resistance to the effects of mitomycin C although this is apparently not a suitable agent for use in selecting for this type of phenotype.

The lack of recombinant clones which secreted either a protease or a DNase was not unexpected as neither of these functions have been reported for B.fragilis BF-1, but it was possibile that the growth conditions for the production of these enzymes was not optimal.

The expression of glutamine synthetase by a recombinant clone was selected as a suitable system for further study, as this enzyme and its regulation are important for the characterization of an organism, and in the well characterised *E.coli* system there is a complex interaction between the regulators of this gene and other nitrogen metabolizing operons.

CHAPTER 5

EXPRESSION AND REGULATION IN E.COLI OF A CLONED B.FRAGILIS GENE FOR GLUTAMINE SYNTHETASE.

SUMMARY

A glutamine synthetase (GS) gene, glnA from B.fragilis was cloned on a recombinant plasmid pJS139 which enabled E.coli glnA deletion mutants to utilize $(NH_4)_2SO_4$ as a sole source of nitrogen. DNA homology was not detected between the B.fragilis glnA gene and the E.coli gene. The cloned B.fragilis glnA gene was expressed from its own promoter, was subject to nitrogen repression in E.coli, but was not able to activate histidase activity in an E.coli glnA, ntrB, ntrC deletion mutant containing the Klebsiella aerogenes hut operon.

5. 1. INTRODUCTION

Glutamine synthetase (GS) has been extensively studied particularly in the Enterobacteriaceae (reviewed by Magasanik 1982). Glutamine is an important amino-acid for the metabolism of nitrogen in all living organisms. It is used as a component for the synthesis of most proteins and as a nitrogen source during the synthesis of numerous other cell components such as other amino-acids, nucleotides, and amino-sugars. The GS enzyme takes part in many of the reactions involving glutamine (Table 6. 1) and is an enzyme of particular importance in metabolism, linking the many catabolic processes producing ammonia and ∝-ketoglutarate with the varied biosynthetic pathways leading to the production of proteins, nucleic acids, complex polysaccharides, and some vitamins. The control of such an enzyme is crucial for co-ordinated cell metabolism, and studies of this enzyme and its control are essential to the understanding of the characteristics of a bacterial species.

The presence of two systems of ammonia assimilation has been reported in B.fragilis and Bacteroides amylophilus, the GS activity was regulated by the availability of ammonia, while the glutamate dehydrogenase pathway continued to function (Yamamoto et al., 1984, and Jenkinson et al., 1979). GS has not been reported from other organisms of the Bacteroides group, although the

known biosynthetic capabilities of these organisms (Varel & Bryant 1974) indicate the probable existence of such an enzyme in most species.

The control of GS production in the Enterobacteriaceae is complex with a variety of different modulators (reviewed by Magasanik, 1982). In E.coli the fully derepressed GS enzyme is produced during growth on glutamate or growth-limiting levels of ammonium salts. These GS levels are significantly reduced when the growth medium contains abundant ammonium salts or adequate quantities of glutamine.

In these bacteria other operons involved in nitrogen metabolism are co-regulated with the structural gene for GS, glnA. This regulation is mediated by the products of the ntrB and ntrC genes which are linked to the glnA gene, and by the product of the unlinked ntrA gene (Garcia et al., 1977, Kustu et al., 1979, Pahel et al., 1979, McFarland et al., 1981). This form of co-regulation has not been found for the GS enzyme of Bacillus subtilis (Fisher et al., 1984, Gardener & Aronson 1984), but has been found for GS genes cloned in E.coli from the chemolithotroph Thiobacillus ferrooxidans (Barros et al., 1985 & 1986).

Multiple forms of GS have been found in *Rhizobium*, one which is similar in character to that found in *E.coli* while the other is heat-labile, not affected by adenylylation, but is repressed by the presence of ammonia. It appears essential for nodulation and the production of nitrogenase (Magasanik, 1982). Evestigneeva & Kaush (1983) report the presence of a third GS from *Rhizobium lupini* bacteroids.

The presence of an ATP-dependent GS from *Selenomonas ruminantium* has been reported which shows no evidence of control by adenylylation and is not active in the γ-glutamyl transferase assay (Smith *et al.*, 1980). *Succinovibrio dextrinosolvens* has been reported as the source of GS which has properties similar to those of the *E.coli* enzyme (Patterson & Hespell 1985).

SECTION 2. MATERIALS AND METHODS

5. 2. 1: BACTERIAL STRAINS AND PLASMIDS.

A list of the bacterial strains used in this chapter are given in Table 5. 1. The derivation of the plasmid pJS139 is described in Chapter 4.

5. 2. 2: BACTERIAL GROWTH MEDIA AND CONDITIONS.

The E.coli strains were grown in Luria Broth or CSH minimal medium supplemented as described in the text (Davis et al., 1980). The formulation and method of preparation of all the media are given in Appendix 2.

5. 2. 3: RESTRICTION MAP OF PLASMID pJS139.

Restriction enzymes were obtained from Amersham International (UK), Bethesda Research Laboratories, Boehringer Mannheim, and New England Nuclear. They were used according to the manufacturers directions. Standard techniques of single and double digests of plasmid DNA with a variety of restriction enzymes was used to establish the map of the plasmid (Maniatis et al., 1982). Restriction fragment sizes were estimated by comparison with molecular size standards of λ-DNA digested with BstEII, HinDIII, or PstI, which were electrophoresed in the same gel.

Methods of electrophoresis are described in Appendix 4.

TABLE 5. 1.

BACTERIAL STRAINS AND PLASMIDS

STRAIN	RELEVANT GENOTYPE	REFERENCE/SOURCE
E.coli ET8051	*glnA*$^-$, *ntrB*$^-$, *ntrC*$^-$, *Ap*^{s}	Tuli *et al.*,1982.
E.coli YMC-10	*glnA*$^+$, *ntrB*$^+$, *ntrC*$^+$, *Ap*^{s}	Backman *et al.*,1981
E.coli YMC-11	*glnA*$^-$, *ntrB*$^-$, *ntrC*$^-$, *Ap*^{s}	Backman *et al.*,1981.
PLASMIDS		
pEcoR251	*Ap*^{r}, *EcoR1*,	M.M.Zabeau, Plant genetic Systems, Ghent, Belgium.
pC_{I857}	Kan^r, Lambda P_R repressor(ts)	M.M.Zabeau, Plant genetic Systems, Ghent, Belgium.
pJS139	*Ap*^{r}, *glnA*$^+$,	This study
pJS139.1	*Ap*^{r}, *glnA*$^-$, (*XhoI* deletion)	This study
pJS139.2	*Ap*^{r}, *glnA*$^+$, (*StuI* deletion)	This study

5. 2. 4: DNA HYBRIDIZATION.

Chromosomal and plasmid DNA were prepared as described in Chapter 4. 2. 4. Chromosomal DNA extracted from E.coli and B.fragilis BF-1 was digested to completion using the restriction endonuclease XhoI and the fragments separated by agarose electrophoresis in tris-actetate buffer. The DNA fragments were transferred to a nitrocellulose membrane (Hybond-N; Amersham, UK) according to Smith & Summers (1980). A [^{32}P]-labelled probe was prepared from purified pJS139 DNA using a nick-translation kit (Amersham, UK) according to the manufacturers directions. The membrane was treated according to the methods of Meinkoth & Wahl (1984) for prehybridization and hybridization. Conditions of hybridizations were such that a 50% homology between the probe and chromosomal DNA should have been detected.

5. 2. 5: PREPARATION OF DELETION DERIVATIVES OF pJS139:

The purified plasmid pJS139 DNA was digested to completion with XhoI or with StuI, and purified by phenol extraction and ethanol precipitation. Each preparation was then ligated (Davis et al., 1980) and used to transform the E.coli YMC-11 glnA$^-$ deletion strain. Transformants were selected on Luria agar containing ampicillin (amp), 125ug/mℓ. Individual clones were streaked onto minimal medium containing $(NH_4)_2SO_4$ as the sole nitrogen source.

Clones derived from each digest were screened by quick plasmid extraction (Ish-Horowitz & Burke, 1981) and agarose electrophoresis after digestion with the appropriate restriction enzymes to show the structure of the plasmid. The map was established of those plasmids which had a putative deletion within the inserted segment of DNA.

5. 2. 6: ASSAY FOR β-LACTAMASE.

The microiodometric method of Sykes & Nördstrom (1972) was used to assay the supernatant fluids of E.coli YMC-11 (pJS139) cultures grown on minimal medium with and without 15mM glutamine. The results were expressed as enzyme units per mg protein.

5. 2. 7: GLUTAMINE SYNTHETASE ASSAY.

GS activity was assayed in crude cell extracts by the γ-glutamyltransferase assay which determines the total GS activity (Bender et al., 1977). Specific enzyme units were expressed as μM glutamyl hydroxamate formed per min per mg protein.

Protein was determined by the dye-binding method of Bradford (1976).

5. 2. 8. REPRESSION OF THE λ-PROMOTER OF pJS139:

The plasmid pC_{I857} encodes the genes for kanamycin resistance and a temperature sensitive repressor of the λ-rightward promoter.

This plasmid was transformed into E.coli (pJS139) and transformants were selected on amp (125ug/mℓ) and kanamycin (20ug/mℓ). Twelve of these transformants were checked by growth on minimal medium at 25°C and 39°C, and by assay of GS produced in minimal medium at the two temperatures.

5. 2. 9: REGULATION OF NITROGEN METABOLISM OPERONS:

Utilization of arginine and low levels of glutamine as sole nitrogen sources (ntr phenotype) was determined by growth on minimal medium containing glutamine (0.5mM) or arginine (15mM) as described by Tuli et al. (1982). Histidase activity was assayed using a modification of the method of Smith et al. (1971).

Exponential phase cells (2mℓ) were well mixed with 0.5mℓ of toluene to make the cells permeable. The cells were collected by centrifugation, resuspended in 0.2mℓ of 0.15M NaCl and stored at -60°C until assayed. For the assay, 20uℓ of sample was mixed with 100µℓ of 1M diethanolamine-HCl buffer (pH 9.4), 10µℓ of freshly made 0.5M reduced glutathione in potassium phosphate buffer (pH 7.4), and 550uℓ of distilled water. After 5min incubation at 37°C, 100uℓ of 0.1M histidine was added, mixed and incubation at 37°C continued for 15min. The reaction was stopped by the addition of 1mℓ of saturated sodium tetraborate. The optical density of the product of the reaction (uracanic acid) was determined at 277nm and the specific activity was expressed as arbitrary units calculated as OD_{277} per mg protein.

SECTION 3. RESULTS

5. 3. 1: ISOLATION OF THE PLASMID pJS139.

The plasmid pJS139 was isolated from a genetic library prepared from the B.fragilis Bf-1 strain, as described in Chapter 4. The presence of a structural gene for GS on the plasmid was confirmed by retransformation of E.coli YMC-11 glnA$^-$, ntrB$^-$, ntrC$^-$ and E.coli ET8051 glnA$^-$, ntrB$^-$, ntrC$^-$ deletion strains. After transformation approximately equal numbers of colonies were obtained on minimal medium (glnA$^+$) and Luria medium containing amp (amp^r).

5. 3. 2: RESTRICTION MAP AND LOCATION OF GS GENE ON pJS139

The restriction map of pJS139 was obtained by complete single or double digestions with restriction endonucleases (Fig. 5. 1). The localization of the DNA region determining the GlnA$^+$ phenotype was determined by the isolation of pJS139 deletion plasmids. The GS gene was located within a 4.2kb fragment of the 8.7kb insert, which was close to the junction of the B.fragilis DNA insert and the pEcoR251 DNA. Excision of a 2.7kb fragment of DNA from this region using XhoI abolished the GlnA$^+$ phenotype (pJS139.1), while excision of a 4.0kb plasmid fragment bounded by StuI restriction sites (pJS139.2) from within the insert, did not cause the loss of the GlnA$^+$ phenotype.

5. 3. 3: THE ORIGIN OF THE GS GENE OF pJS139.

The origin of the 8.7kb insert in pJS139 was determined by Southern blotting and DNA hybridization between B.fragilis chromosomal DNA and [^{32}P]-labelled pJS139 (Fig. 5. 2).

The plasmid pJS139 has two XhoI fragments of approximately 9.3 and 2.7kb. The 2.7kb fragment is internal to the putative B.fragilis glnA insert, but is adjacent to the junction between the vector and insert DNA. Therefore [^{32}P]-pJS139 was hybridized to both B.fragilis chromosomal DNA and pJS139 digested with XhoI and a positive hybridization signal was detected in both digests (Fig. 5. 2B, lanes 2 and 5). A second band of hybridization of approximately 10kb was also observed. No DNA hybridization was detected between [^{32}P]-pJS139 and a XhoI digest of chromosomal DNA from the E.coli HB101 glnA^{+} strain.

TABLE 5. 2.

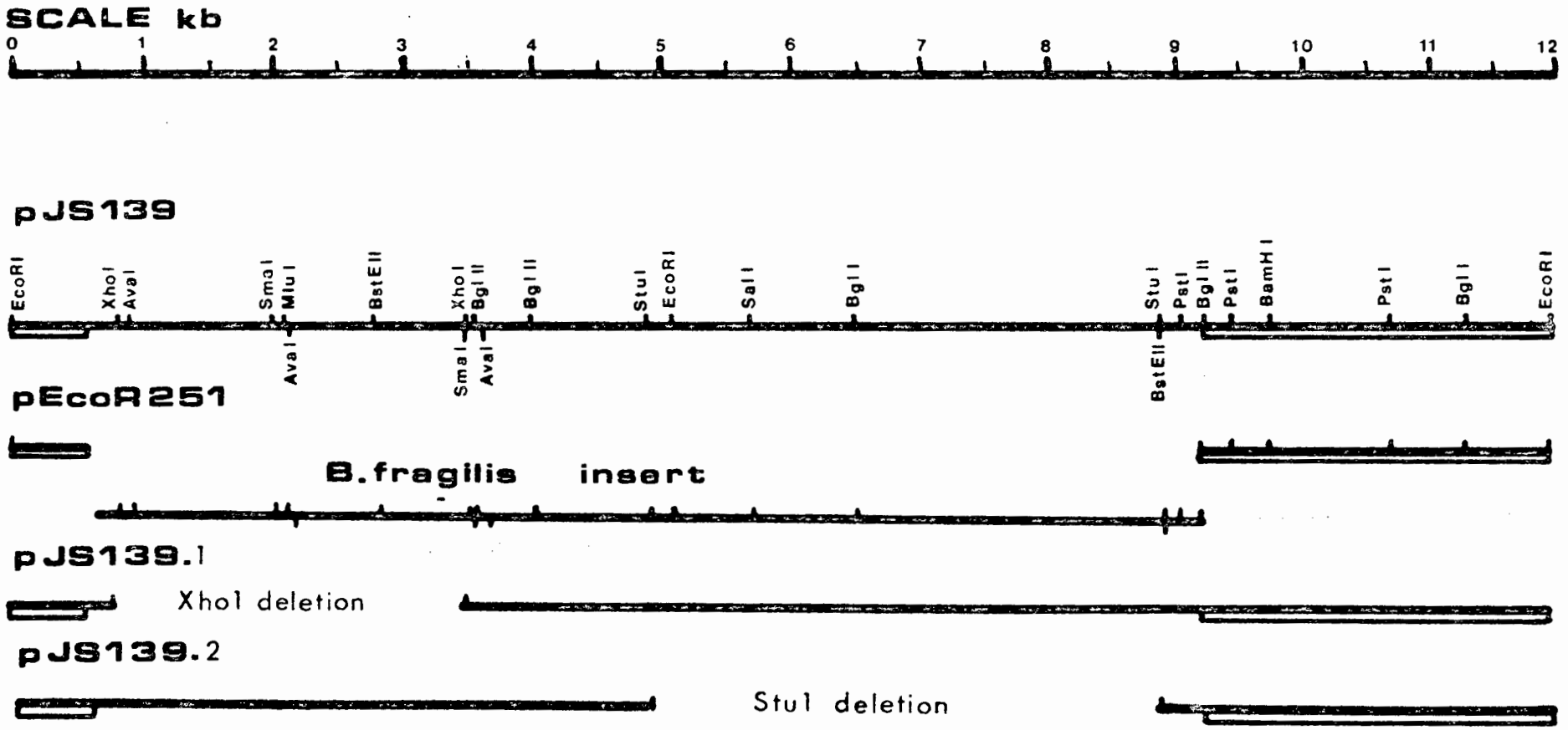
Restriction fragment lengths obtained by digestion of pJS139 with various restriction enzymes, estimated by agarose electrophoresis.

ENZYME .NAME	RECOGNITION SEQUENCE	FRAGMENT LENGTH
AvaI	5'-C*PyCGPuG-3'	1.25, 1.5, 9.25
BamHI	5'-G*GATCC-3'	12
BglI	5'-GCCNNNN*NGGC-3'	4.8, 7.2
BglII	5'-A*GATCT-3'	0.45, 5.2, 6.4
BstEII	5'-G*GTNACC-3'	5.8, 6.15
EcoRI	5'-G*AATTC-3'	5.1, 6.9
MluI	5'-A*CGCGT-3'	12
PstI	5'-CTGCA*G-3'	0.4, 1.8, 10.2
SalI	5'-G*TCGAC-3'	12
SmaI	5'-CCC*GGG-3'	1.5, 10.5
StuI	5'-AGG*CCT-3'	4.0, 8.0
XhoI	5'-C*TCGAG-3'	2.7, 9.3

*=cleavage point.

FIGURE 5. 1.

RESTRICTION MAP OF pJS139 AND ITS DELETION DERIVATIVES.



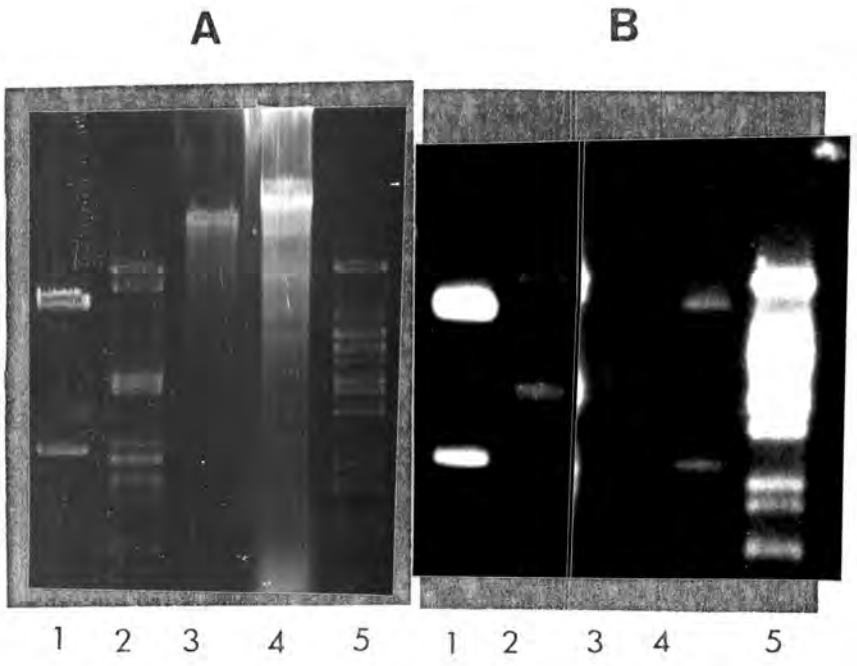


FIGURE 5. 2. Hybridization of [^{32}P]-pJS139 to B.fragilis and E.coli chromosomal and plasmid digests of DNA. A: Approximately 20ug of DNA from chromosomal and 3ug from plasmid restriction digests was electrophoresed in a 0.8% Biorad agarose gel in tris-acetate buffer and stained with ethidium bromide. Lane 1: pJS139-XhoI, 2: λ-PstI, 3: E.coli HB101-XhoI, 4: B.fragilis BF-1-XhoI, 5: λ-BstEII. B: Autoradiograph of a Southern blot of the gel in A following hybridization. Lanes 1 and 2: 24h exposure; lanes 3, 4 and 5: 48h exposure. The hybridization probe was [^{32}P]-pJS139, to which [^{32}P]-labelled λ-DNA was added as an internal reference.

5. 3. 4. EXPRESSION AND REGULATION OF GS ACTIVITY.

GS activity was readily detectable in cell extracts of E.coli YMC-11 containing pJS139 and in E.coli YMC-10 grown under nitrogen limiting conditions, while no GS activity was detected in extracts of E.coli YMC-11 or ET8051 grown under any conditions (Table 5. 3).

GS activity in E.coli YMC-11 containing pJS139 was partly repressed by ammonium salts but fully repressed by glutamate and glutamine. GS activity of the E.coli YMC-10 wild type strain was partly repressed during growth in medium containing glutamine or ammonium salts but was fully repressed if glutamine was present (Table 5. 3).

5. 3. 5. REPRESSION OF THE λ-RIGHTWARD PROMOTER.

Clones of E.coli YMC-11 (pJS139, pC_{I857}) and E.coli YMC-11 (pJS139) grew equally well on minimal medium plates at each temperature (23°C and 39°C). In minimal medium approximately equal quantities of GS activity per mg protein, was detected (an average of 0.35 and 0.43 AU for the different temperatures respectively).

5. 3. 6. REGULATION OF NITROGEN METABOLISM OPERONS.

In E.coli and Salmonella typhimurium the ntrB and ntrC genes which have been shown to be closely linked to the GS gene, regulate not only the GS gene but also a high affinity glutamine and a high affinity arginine transport system (Kustu et al., 1979). These ntr genes have also been shown to activate the histidine operon (Magasanik, 1982).

Table 5. 3.

RELATIVE LEVELS OF GS AND HISTIDASE ACTIVITY

Strains were grown in CSH glucose minimal medium (MM) with 15mM glutamate as nitrogen source and then diluted in minimal medium supplemented as follows: H: 15mM glutamate, 15mM glutamine. L: 15mM glutamate, 0.15mM glutamine. N: 1g/l $(NH_4)_2SO_4$ in CSH minimal medium, F: Nitrogen free CSH minimal medium. Samples were assayed after 3h incubation at 37°C in shake flasks. GS enzyme activity is expressed as umol glutamyl hydroxamate produced per min per mg protein, histidase activity is expressed as arbitrary units as defined in the text. Standard errors of the means were 5 to 10% of the reported values.

	GLUTAMINE SYTHETASE U				HISTIDASE AU		
MEDIUM / STRAIN	H	L	N	F	H	L	F
YMC-10 *glnA*$^+$,*ntr*$^+$	0.08	1.24	1.1	2.49	12.55	24.25	32.85
YMC-11 *glnA*$^-$,*ntr*$^-$	0	0	0	0	6.35	6.65	15.55
YMC-11pJS139 *glnA*$^+$,*ntr*$^-$	0	0.05	1.25	4.15	7.65	6.6	14.3

The ability of the various E.coli strains to grow on minimal medium containing low levels of arginine and glutamine was determined. The ET8051 and YMC-11 glnA deletion strains were unable to grow on these media. When these strains contained the recombinant plasmid pJS139 a very weak growth was obtained (Table 5. 4).
In comparison the YMC-10 glnA$^+$, ntrB$^+$, ntrC$^+$ strain grew well under these conditions.

The E.coli strains YMC-10 and YMC-11 carry a Klebsiella aerogenes hut operon (Tuli et al., 1982) which has a hutC mutation resulting in high basal levels of histidase. The results in Table 5. 4 show that this enzyme level was increased in the YMC-10 strain by the ntrB and ntrC co-regulation of the hut operon (Tuli et al., 1982) in low concentrations of glutamine, but this was not observed with the YMC-11 glnA, ntrB, ntrC or the YMC-11(pJS139) glnA$^+$ strains. Increased levels of histidase were observed with all strains when cells were suspended in nitrogen free minimal medium.
The β -lactamase content of E.coli YMC-11(pJS139) cells grown in minimal medium with and without glutamine was similar (35.1 and 37.8 units per mg respectively).

TABLE 5. 4.

Growth of the different E.coli strains on minimal medium plates containing different nitrogen supplements.

Supplemented minimal medium plates were inoculated with suspensions of the different E.coli strains and incubated for 24h at 37°C.

Key: -, no growth; ±, slight growth; +, good growth.

E.coli STRAIN	YMC-10	YMC-11	ET8051	YMC-11 (pJS139)	ET8051 (pJS139)
MEDIUM SUPPLEMENT					
None	±	-	-	-	-
20mM $(NH_4)_2SO_4$	+	-	-	+	+
0.5mM glutamine	+	-	-	±	±
10mM arginine	+	-	-	-	-
0.5mM glutamine & 10mM arginine	+	-	-	±	±
Luria medium &:					
Amp (125µg/mℓ)	-	-	-	+	+
Streptomycin (40µg/mℓ)	-	-	+	-	+
None	+	+	+	+	+

SECTION 5. DISCUSSION

The putative B.fragilis BF-1 GS gene carried by the recombinant plasmid pJS139 was shown to be derived from the B.fragilis BF-1 strain and to be functional in E.coli glnA$^-$ deletion mutants. This is the first report of expression of a gene from the medically important obligate anaerobe B.fragilis cloned in E.coli.

The origin of the glnA gene was confirmed by DNA hybridization and it directed the synthesis, in E.coli glutamine auxotrophs, of a B.fragilis GS which was enzymatically active.

The expression of the cloned GS appears to be from a promoter contained within the inserted B.fragilis DNA segment of pJS139 as there have been no reports of a pEcoR251 plasmid promoter regulated by nitrogen levels. In addition E.coli YMC-11 (pJS139) containing the pC_{I857} kan^r and λ-repressor(ts), produced approximately the same levels of GS per mg protein when incubated in nitrogen free minimal medium at 23°C and at 39°C, which indicated that the cloned B.fragilis glnA gene was not expressed from the rightward λ-promoter present on the vector.

Data presented in chapter 8 indicate that the size of the GS polypeptide in B.fragilis is the same as that of the cloned GS.

The B.fragilis glnA gene was subject to nitrogen repression in E.coli. The regulation by nitrogen affected the glnA gene and was not due to an increase in plasmid copy number as a result of growth in the different media.

β-lactamase, produced by E.coli YMC-11 (pJS139), assayed after growth on the different nitrogen media did not show any variation in the levels of activity of the enzyme in the samples assayed. This enzyme is constitutively produced by the plasmid vector pBR322 component, and levels should reflect plasmid copy number.

The regulation of the cloned B.fragilis glnA gene differed from the regulation of the E.coli glnA gene in the wild type strain. The cloned B.fragilis glnA gene was repressed by glutamate whereas the E.coli gene was expressed in the presence of glutamate. Since the glnA gene on pJS139 was regulated by nitrogen it is suggested that some nitrogen regulatory activities are located on pJS139 but that these activities are not directly analogous to the ntrB and ntrC system of the enteric bacteria.

In comparison with the E.coli wild type strain the cloned B.fragilis DNA fragment only enabled weak growth of the E.coli glnA, ntrB, ntrC deletion mutant on media containing arginine or low levels of glutamine as sole sources of nitrogen and it was not able to activate the Klebsiella hut operon in E.coli YMC-11.

In order to study this B.fragilis gene product further the protein encoded by the plasmid was purified and its characteristics studied in Chapter 6.

CHAPTER 6.

PURIFICATION AND PROPERTIES OF THE CLONED GS ENZYME.

SUMMARY

The glutamine synthetase (GS) encoded by a cloned B.fragilis gene was extracted and purified from the E.coli YMC-11 (pJS139) strain. Purification was by differential polyethylene glycol precipitation and Sephacryl chromatography.

SDS-PAGE of the purified enzyme showed a single polypeptide subunit with M_r of approximately 74 000. Chromatography of the enzyme on a calibrated S-400 column indicated that the active holoenzyme had an approximate M_r of 490 000. This was confirmed by non-denaturing pore gradient electrophoresis in a polyacrylamide gel gradient. Electron micrographs indicated a typical hexagonal structure for the enzyme, but from the M_r determinations it is concluded that the enzyme which has been purified is probably a hexamer, unlike the dodecameric enzymes reported for GS enzymes from other Eubacteria.

This purified enzyme was active in both the γ-glutamyl transferase and the forward transferase assay for GS.

SECTION 1. INTRODUCTION

The GS enzyme has been purified from many microorganisms; that from E.coli has been best characterized and although the GS from other organisms differ in details, there are many common features. The structure and subunit-subunit interactions of purified unadenylylated GS from S.typhimurium has been described and an atomic model determined which suggests the function of the subunit interface in catalysis and regulation of the enzyme (Almassy et al. 1986).

The GS enzyme isolated from E.coli (reviewed by Stadtman & Ginsburg, 1974) consists of 12 polypeptide subunits each of M_r 50000, these are combined in a double hexagonal "doughnut" shaped structure. This structure has been found in other organisms although the reported M_r of the subunits varied from 50000 to 62000.

In E.coli GS is regulated by at least six distinct mechanisms:

1. The repression of enzyme synthesis in response to the concentration of metabolites in the growth medium (discussed in Chapter 5).
2. Cumulative feed-back inhibition by the many end products of glutamine metabolism.
3. Interconversion of active and inactive forms of the enzyme by changes of concentration of divalent cations.
4. Variation of the enzyme's catalytic potential and it's sensitivity to divalent cations by the enzymatic covalent adenylylation and deadenylylation of the enzyme.

5. The cascade control of the enzymatic adenylylation and deadenylylation reactions; between 0 and 12 adenylyl groups can be bound by the molecule, one by each subunit.

6. Modulations of enzymatic activity by variation in relative concentrations of divalent cations and nucleotide triphospates.

Several reactions which require divalent cations and energy in the form of ATP, may be catalysed by E.coli GS (Table 6. 1). These reactions all concern the transfer of amino groups from glutamate or glutamine or sometimes ammonia.

The activity of GS can be assessed in crude cell extracts using either the γ-glutamyltranferase (GGT) assay or the biosynthetic forward transferase assay (Bender et al., 1977). In E.coli and Klebsiella aerogenes the GGT assay, carried out in low Mg^{2+} concentrations, at the iso-activity point, estimates the total quantity of GS present irrespective of the conformation of the enzyme or its adenylylation state.

During extraction and purification the state of adenylylation of the E.coli enzyme can be stabilized using the compound N-cetyl-NNN-trimethylammonium bromide (CTAB) (Bender et al.,1977). This is important as the enzyme may become adenylylated during extraction, as reported by Engelhardt & Klemme (1982) for Rhodopseudomonas sphaeroides a phototrophic organism.

The GS enzymes from Gram-positive bacteria are not controlled by the covalent binding of adenylyl groups to the enzyme subunits although the general structure is the same (Deuel et al., 1970).

TABLE 6. 1.

REACTIONS CATALYSED BY E.COLI GLUTAMINE SYNTHETASE

From Stadtman and Ginsburg (1974)
All the reactions require divalent metal cations as cofactors.

$ATP + glutamate + NH_3 \longrightarrow glutamine + ADP + P_i$

$ATP + glutamate + NH_2OH \longrightarrow \gamma\text{-glutamylhydroxamate} + ADP + P_i$

$ATP + glutamate \longrightarrow pyrrolidine\ carboxylate + ADP + P_i$

$Glutamine + NH_2OH \xrightleftharpoons[nucleotide,Pi\ or\ arsenate]{} \gamma\text{-glutamylhydroxamate} + NH_3$

$ATP + methionine\ sulphoximine + GS \longrightarrow GS\text{-methionine sulphoximine-P.ADP}$

$ATP + H_2O \longrightarrow ADP + P_i$

$Glutamine + H_2O \xrightarrow[ADP,\ arsenate]{} glutamate + NH_3$

$Glutamylhydroxamate \xrightarrow[ADP,\ arsenate]{} NH_2OH + glutamate.$

The GS cloned and purified from Clostridium acetobutilicum by Usdin et al., 1986, was very susceptible to inhibition by divalent metal cations, and was composed of 12 subunits with M_r of 50 000.

The reported structure for GS purified from Clostridium pasteurianum was 20 subunits of M_r 50 000 each which resulted in an M_r for the holoenzyme of 1×10^6 (Krishnan et al., 1986).

The archaebacterium, Methanobacterium ivanovi, has been reported to possess a GS with 12 subunits, but it may not be controlled by adenylylation/deadenylylation reactions common in the Eubacteria (Bhatnagar et al., 1986).

GS from eukaryotic cells has an 8 subunit structure which differs from bacterial GS in several respects (Carlson & Chelm, 1986).

Purification of the GS enzyme has been achieved in a number of different ways, often exploiting the relatively large size of the molecule. A method commonly used is purification by differential centrifugation (Streicher & Tyler 1980, Bodasing et al., 1985), another is the precipitation from solution in paracrystalline arrays using low concentrations of Zn^{2+} (Stadtman & Ginsburg 1974). Polyethylene glycol may also selectively precipitate large molecules from complex solutions, and gel-filtration using Sephacryl S1000 is able to separate molecules on the basis of molecular size.

SECTION 2. MATERIALS AND METHODS

6. 2. 1.

EXTRACTION AND PURIFICATION OF THE pJS139 GENE PRODUCT

The enzyme was extracted from E.coli YMC-11 (pJS139) cells which had been grown overnight in Luria Broth and then diluted into an equal volume of nitrogen-free CSH minimal medium and shaken at 37°C for 2h. The stabilizer of GS, CTAB, was added to the culture to a final concentration of 0.1mg/mℓ and incubation continued for 10min. The cells were collected by centrifugation and resuspended in 1/100th of the culture volume in extraction buffer (Imidazole-HCl; 50mM, $MgCl_2$; 20mM, phenylmethylsulphonyl fluoride; 0.1mg/mℓ, and 2-mercaptoethanol; 20mM, pH 7.15) and sonicated (Heat systems USA.) to disintegrate the cells. After centrifugation at 10000 X g for 20min, the supernatant was kept on ice and NaCl was added to 0.1M and PEG (6000 MW) to 4% (w/v). After 4h the mixture was centrifuged and PEG added to the supernatant to bring it to 6%(w/v), the mixture was kept at 4°C overnight.

The precipitate was collected, dissolved and loaded onto a Sephacryl S1000 (Pharmacia Corp.) 250 X 10mm column, equilibrated and eluted with column buffer (Imidazole-HCl; 10mM, KCl; 1% (w/v), pH7.15) (C buffer) at 0.2ml/min. Fractions (1mℓ) were collected and assayed for GS activity and analysed by SDS-PAGE (Laemmli, 1970, O'Farrell, 1975). A centrifuged (170000 X g for 1h) sample of purified GS was stained with sodium phosphotungstate and examined in a transmission electron microscope.

6. 2. 2. ASSAY FOR GS ACTIVITY:

GS was assayed by the ɣ-glutamyl transferase (GGT) assay according to the method of Bender et al. (1977). The optimum conditions for the assay of cloned B.fragilis GS were determined as described in chapter 7. Specific enzyme activity was expressed as µM of glutamylhydroxamate formed per min, per mg protein. Protein was determined using the dye-binding method of Bradford (1976).

6. 2. 3. PREPARATION OF ANTIBODIES TO THE PURIFIED GS:

Rabbits were immunized by intra-muscular injection of approximately 200ug of the purified GS protein on days 1, 3, 7, 14 and 21, and serum was collected on day 0 and day 24. Ouchterlony immunodiffusion plates were prepared (Weir, 1973) using 1% agarose in tris-borate buffer (Appendix 2). After precipitin bands were apparent, the agarose gel was washed for 4 h in running water, dried onto Gelbond film (FMC Corp.), stained with aceto-orcein (Orcein; 2 g, in 100 mℓ 50% (v/v) acetic acid) and destained in absolute alcohol for photography.

6. 2. 4. SEPHACRYL S-400 COLUMN CHROMATOGRAPHY:

A calibrated Sephacryl (Pharmacia corp.) S-400 column of 10 X 400 mm was used according to the manufacturers instructions to estimate the M_r of the purified GS. The gel was equilibrated in the same buffer as used for S-1000 chromatography and eluted at a flow rate of 0.1 ml min^{-1} and 0.5 ml fractions were collected and assayed for GS activity using the GGT assay. All operations were carried out at a temperature of 4 - 8 °C.

6. 2. 5. SIZE ESTIMATION BY PORE GRADIENT ELECTROPGORESIS.

Pore gradient (PG) electrophoresis in a 4 - 30% (w/v) non-denaturing polyacrylamide gradient was carried out according to the instructions accompanying the Pharmacia HMW molecular weight marker kit. The electrophoresis buffer was 0.09 M Tris-borate-EDTA, pH 7.5. Electrophoresis was for 7 h at 330 v (2310 vh) after which the gel was divided and one part was stained with coomasssie brilliant blue and the three lanes in the second part, which had been loaded with GS, were cut into 5 mm slices, the gel crushed with 1.0 ml C buffer and assayed for GS activity.

SECTION 3. RESULTS

6. 3. 1. PURIFICATION OF THE CLONED glnA GENE PRODUCT:

The specific activity of the GS increased during the purification and resulted in a 280-fold purification of the enzyme (Table 6. 2). As a result of the increase in total specific activity an apparent 100% recovery of the enzyme was obtained. The GS activity was not precipitated from the crude cell lysate by 4% (w/v) PEG but was by 6% (w/v) PEG (Fig. 6. 1). An increase in total GS activity following 6% PEG precipitation was observed. This resulted in an apparent overall recovery of 214% at this stage. This increase was due to the removal of an inhibitor from the crude lysate during purification as addition of crude material to purified GS resulted in an 80% decrease in specific activity. The identity of this inhibitor has not been determined.

It was not possible to purify the E.coli YMC-10 GS using this method as the GS activity was not precipitated at 6% (w/v) PEG. The E.coli GS activity was only precipitated at between 8 and 10% PEG when large numbers of other cellular proteins co-precipitated resulting in no overall purification.

The GS activity eluted as a single peak from S-1000 gel chromatography and also from S-400 which has a more appropriate fractionation range (Fig. 6. 3.).

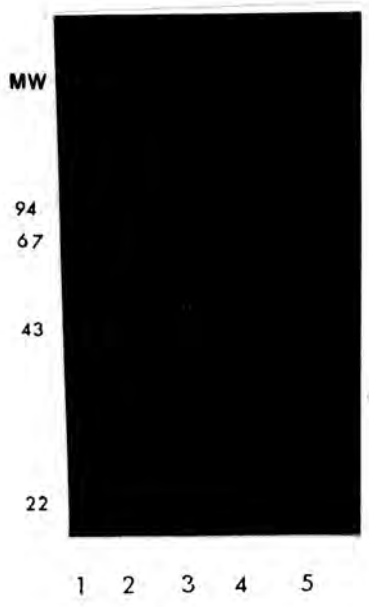


FIG. 6. 1. SDS-PAGE of samples taken at various steps during GS purification.

Approximately equal volumes of sample were electrophoresed in a 10% polyacrylamide gel and stained with coomassie blue. Lane 1: M_r markers X 10^3, 2: S-1000 GS activity peak (less than 1µg), 3: PEG precipitate (5µg), 4: 6% (w/v) PEG supernatant (5µg), 5: Whole cell lysate (7µg) of E.coli YMC-11 (pJS139).

TABLE 6. 2:

PURIFICATION OF THE CLONED B.fragilis GS ENZYME FROM E.coli YMC-11(pJS-139)

Total GS activity was determined by the - glutamyl transferase assay. The details of the purification and assay procedures are described under Methods. GS activity is expressed as units equal to umol of glutamylhydroxamate formed per min, per mg protein. Fraction 1 and fraction 5 correspond to lanes 5 and 1 of Fig. 6. 1.

FRACTION NUMBER	1 Ultra-sonic extract	2 4% PEG spnt.	3 6% PEG spnt.	4 6% PEG ppt	5 S-1000 peak fraction
VOLUME ml	25	25	25	1.5	1.0
TOTAL PROTEIN mg	175	152	131	36	0.7
TOTAL GS ACTIVITY u	62.5	59.4	10.2	133.9	71.0
SPECIFIC ACTIVITY u/mg	0.36	0.39	0.08	3.72	101.4
PURIFICATION FOLD	1.0	1.09	0.22	10.41	284.3
PERCENT RECOVERY	100	95	16	214	114

6. 3. 2: PHYSICAL CHARACTERISTICS OF THE ENZYME:

The M_r of the GS subunit was estimated by comparison with molecular size markers on SDS-PAGE (Fig. 6. 2) to be approximately 74000. This corresponded to an increased band of protein seen in whole cell lysates of E.coli YMC-11 (pJS139) compared to lysates from E.coli YMC-11 glnA$^-$ cells. There was a relatively small quantity of total protein in the YMC-11 lysate, which was due to the lack of growth in the medium used to induce the GS from E.coli YMC-11 (pJS139).

No other protein bands were visible on coomassie blue stained gels of the purified preparation which indicated that the visible band was the GS enzyme.

The M_r of the holoenzyme estimated by S-400 chromatography (Fig.6. 3.) and PG electrophoresis (Fig. 6. 4.) was approximately 490 000 although these methods are unlikely to be accurate to more than about 2% (or M_r 10000). The fraction from the S-400 column which showed GS activity gave an equivalent band on PG electrophoresis. It was not possible to demonstrate GS activity in crushed gel slices from the PG electrophoresis, possibly due to the EDTA in the gel buffers.

6. 3. 3: ELECTRON MICROSCOPY OF PURIFIED GS:

Electron microscopy showed ring shaped structures which were similar to published electron micrographs (Stadtman & Ginsburg, 1974, Engelhardt and Klemme; 1982) of purified GS from other micro-organisms (Fig. 6. 4). However no tetragonal structures were seen. These are indicative of side views of the double-layered ring structures of the E.coli dodecameral GS enzyme.

6. 3. 3: IMMUNOLOGICAL CHARACTERISTICS OF THE ENZYME:

Rabbit antibodies prepared as described gave a single band in an Ouchterlony plate and showed homology to a single protein in a crude extract of the E.coli YMC-11 (pJS139) strain, but no cross reaction with cell extracts from E.coli YMC-10 glnA$^+$ or YMC-11 glnA$^-$ cultures was observed (Fig. 6. 6). Serum from the rabbit taken at day 0 did not produce precipitin lines with any of the samples. The antiserum did not inhibit the activity of the enzyme in the GGT assay.

The antiserum was used to demonstrate the presence of the GS polypeptide in B.fragilis BF-1 by western blotting, and this is described in chapter 8.

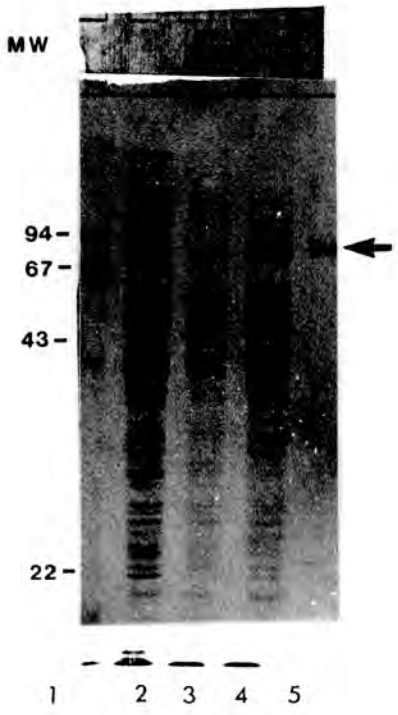


Fig. 6. 2. SDS-PAGE of cell lysates and purified GS.

Samples of lysed cells which had been suspended in minimal medium for 2h before lysis were electrophoresed on a 10% polyacrylamide gel and stained with coomassie blue. The samples were reduced and treated with SDS before electrophoresis to remove secondary and tertiary structures.

Lane 1, M_r markers X 10^3; 2, E.coli YMC-10 wt (13µg); 3, E.coli YMC-11 glnA$^-$ (5µg); 4, E.coli YMC-11 (pJS139) (8µg), 5, purified GS from Sephacryl-S1000 (approx. 1µg).

The arrow indicates the putative GS polypeptide.

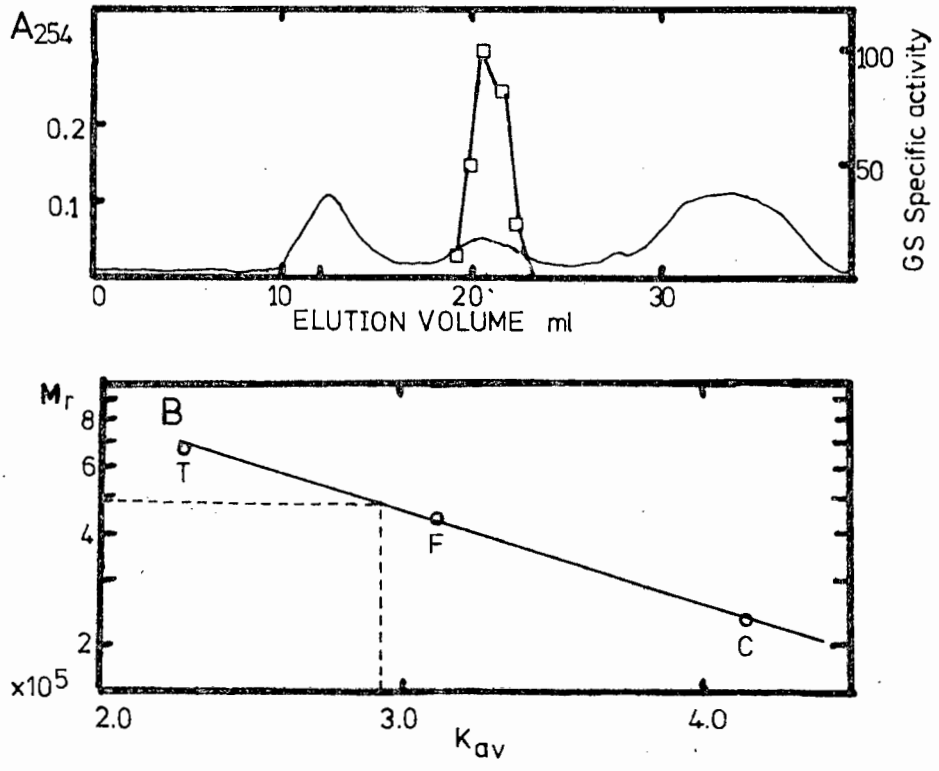


Fig. 6. 3. GEL-FILTRATION CHROMATOGRAPHY OF GS

A: **Elution profile of a sample of crude GS from a S-400 column.**
A sample of the 6% PEG precipitate was run on a S-400 column and the A_{254} continuously determined (---). The GS activity (□-□) of fractions was determined by the GGT assay and expressed as umol glutamyl hydroxamate formed per min, per mg protein.

B: **Estimation of the GS holoenzyme size by gel-filtration.**
The Sephacryl S-400 column was calibrated using Pharmacia HMW proteins, Thyroglobulin(T) M_r 670 000, Ferritin(F) M_r 440 000, Catalase(C) M_r 232 000. A sample of purified GS was run under the same conditions. The K_{av} was calculated using the formula:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Where V_e = the sample elution volume, V_o = the void volume, and V_t = the total bed volume of the column.

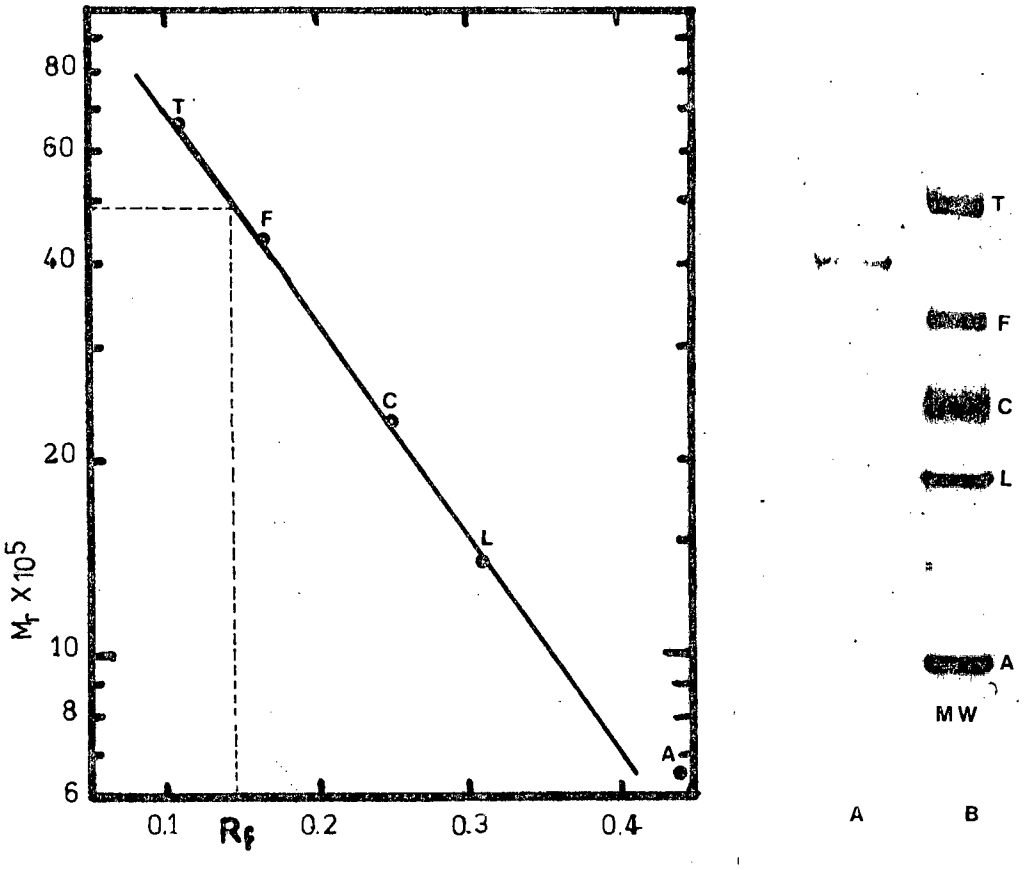


Fig. 6. 4.

Estimation of the GS holoenzyme molecular size by Pore gradient electrophoresis.

A 4 - 30 %(w/v) polyacrylamide gradient gel in Tris-borate-EDTA buffer was electrophoresed for 2300 Vh, and stained with coomassie brilliant blue. The relative mobilities of the marker proteins, Thyroglobulin (670000), ferritin (440000), catalase (232000), lactic dehydrogenase (140000), and bovine albumin (67000) were used to plot a graph from which the size of the GS protein band (490000) was extrapolated. These are the averaged results of three experiments.

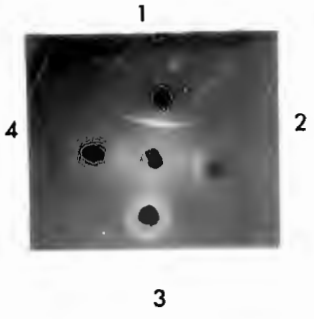


Fig. 6. 5.

Immunodiffusion of cell lysates vs antiserum to GS enzyme extracted and purified from E.coli YMC-11 (pJS139).

The gel contained 1% (w/v) agarose in 0.1M tris-borate buffer (pH 7.8) with 20µℓ per well of antiserum in the centre well and 1, Purified cloned GS; 2, Cell lysate of E.coli YMC-11 (pJS139); 3, Cell lysate of E.coli YMC-10 glnA$^+$; 4, Cell lysate of E.coli YMC-11 glnA$^-$. The gel was stained with aceto-orcein. The halo around sample 3 is due to the higher protein concentration of this sample relative to the other samples.

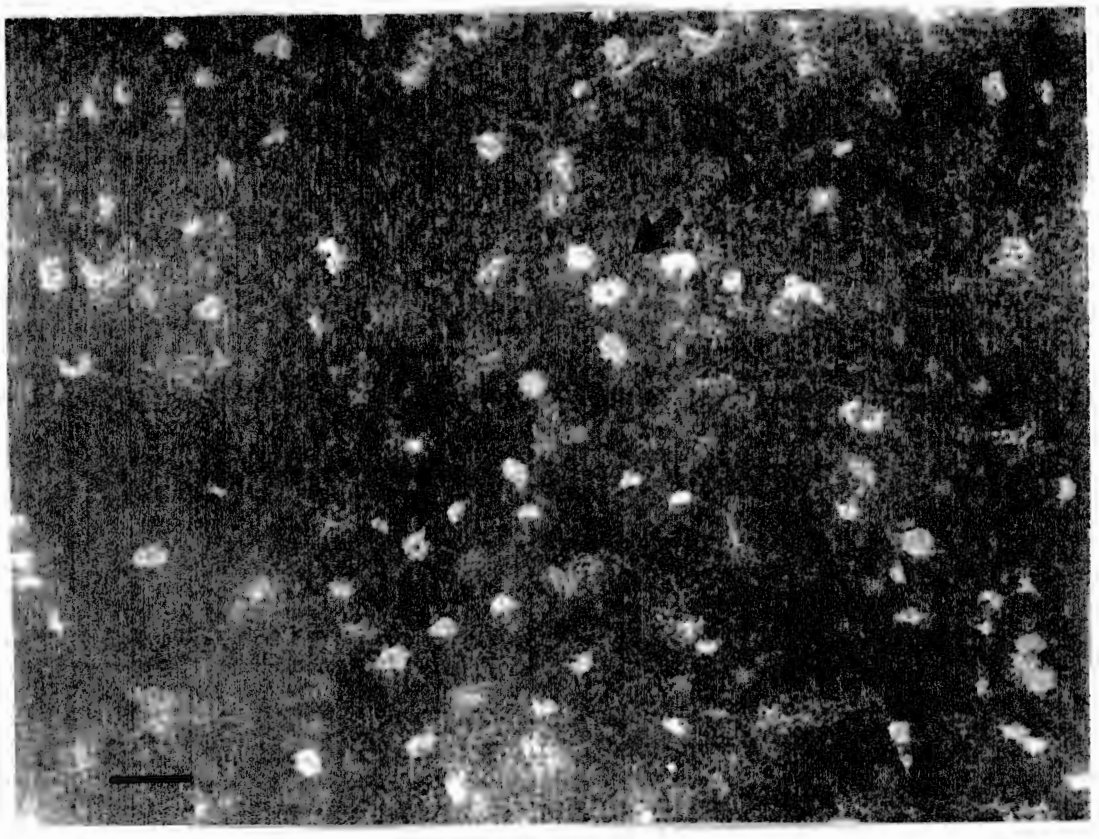


FIG. 6. 6. Electron micrograph of purified GS.

The purified concentrated sample was spread on a carbon coated grid and stained with sodium phosphotungstate and examined at a magnification of 50000. The magnification in the photograph is 250000 and the diameter of a typical molecule is approximately 12nm. The bar indicates 40nm and the arrows typical "doughnut" shaped molecules.

SECTION 4. DISCUSSION

An enzymatically active GS protein was purified from E.coli YMC-11 which contained the recombinant plasmid pJS139. There was only one major band visible on SDS-PAGE, and on Ouchterlony immunodiffusion, and on PG electrophoresis of samples with known GS activity, and it is concluded that this was the GS protein.

The subunit M_r was approximately 74000. The large apparent size of the GS sub-unit is a unique feature of this enzyme. If the enzyme were the typical dodecamer reported for other GS molecules, then the particle weight for the undissociated GS would be approximately 9×10^5. which would be considerably larger than that reported for other bacteria. E.coli, B.subtilis, R.japonicum and Vibrio alginolyticus have subunits with M_r of 50000, 56000, 60000 and 62000 respectively (Stadtman & Ginsburg, 1974; Deuel et al., 1970; Bhandari et al., 1983; Bodasing et al., 1985).

However the evidence from the gel filtration chromatography and PG electrophoresis of the active holoenzyme indicates that the enzyme is not a typical dodecamer, but is probably composed of six sub-units to give a theoretical total M_r of 450000, which is close to the observed value of approximately 490000.

There was a possibility that during the cloning, DNA rearrangements or gene fusions might have created a hybrid gene consisting of other polypeptide fragments and the B.fragilis GS gene which, while still active, may have a much larger size than the native B.fragilis polypeptide subunit.

However the DNA hybridization experiments in chapter 5 indicated the integrity of the cloned gene, and evidence in chapter 8 indicated that the sub unit M_r of GS from B.fragilis was similar to that of the cloned B.fragilis glnA gene product extracted from E.coli YMC-11 (pJS139).

The identity of the GS inhibitor present in crude cell extracts of E.coli YMC-11 has not been determined.

The activity of the cloned enzyme in the γ-glutamyl transferase reaction is described in the next chapter.

CHAPTER 7.

THE ASSAY AND CONTROL OF THE CLONED GS ENZYME.

SUMMARY.

The GS enzyme encoded by a cloned B.fragilis gene in the E.coli YMC-11 (pJS139) strain was studied with regard to its activity in the γ-glutamyl transferase (GGT) and the forward transferase assays. In these assays the GS has optimum activity at a temperature of 45°C and at a pH of between pH 6.5 and pH 7.0.

The cloned enzyme was not controlled by the adenylylation/deadenylylation system, common to the majority of Gram-negative Eubacteriales, but there was evidence for some form of control.

Cloned GS extracted from ammonia shocked cells showed enhanced activity in the presence of low additional concentrations of Mn^{2+} (0.25 mM) in the GGT assay, while this was not the case for GS extracted from cells grown under nitrogen limiting conditions. Both forms of enzyme were inhibited by the presence of 10 mM Mg^{2+} in the GGT assay.

Snake venom phosphodiesterase I treatment of both forms of the enzyme led to a reduction in activity which was not altered by the addition of 0.25 mM Mn^{2+} or 10 mM Mg^{2+} to the treated enzyme.

It appeared that nucleotidylation or phosphorylation of the enzyme was required for its activity, and that the enzyme was inactivated by the removal of these groups.

The activity of the cloned GS in the GGT assay was inhibited by various end products of glutamine metabolism but not by the addition of AMP.

SECTION 1 INTRODUCTION

The factors affecting the control of E.coli GS enzymatic abilities in in vitro assays have been reviewed by Stadtman and Ginsberg (1974). The adenylylation-deadenylylation control of the enzyme is affected by enzymes which, in growth conditions of ammonia excess, sequentially covalently attach adenosine 5'-monophosphate moieties to the GS molecule. This adenylylation results in the reversible inactivation of the enzyme.

Bender et al. (1977) described two assays which reveal the adenylylation state of the enzyme. These are the forward transferase (FT) assay which measures the biosynthetic capacity of the GS, and the γ-glutamyl transferase (GGT) assay, which measures the reverse reaction. In E.coli and K.aerogenes the FT and GGT assays show that unadenylylated GS differs from the adenylylated form in several ways. The unadenylylated GS is stimulated in the presence of 60 mM Mg^{2+} and inhibited by 0.3 mM Mn^{2+} only in the presence of adenosine monophosphate and a feedback inhibitor, while fully adenylylated GS is inactive in the presence of 60 mM Mg^{2+} or any combination of AMP or ADP, with Mn^{2+} or a feedback inhibitor. The enzyme activity assayed at different pH values shows striking changes in most Gram-negative bacteria. In the presence of 0.3 mM Mn^{2+}, E.coli GS extracted from cells which have been grown under nitrogen limiting conditions, and can be shown to be free of adenylyl groups, has an optimum GGT activity at about pH 8.0.

Enzyme from ammonia shocked cells, where the adenylylation approaches twelve, has an optimum GGT activity at pH 6.9.

At an intermediate pH, termed the iso-activity point, the GGT activity is the same for both the adenylylated and the deadenylylated GS enzyme. For E.coli this iso-activity point is pH 7.15.

A variety of intermediate states of the enzyme are possible depending on the degree of adenylylation.

The adenylylation state of the purified enzyme can also be determined spectro-photometrically (Stadtman & Ginsberg; 1974).

The complete deadenylylation of the enzyme can be accomplished using snake venom phosphodiesterase I (SVP).

During extraction and purification the state of adenylylation of the E.coli enzyme can be stabilized using the compound N-cetyl-NNN-trimethylammonium bromide (CTAB) (Bender et al.,1977) as the enzyme may in certain bacteria become adenylylated during extraction (Engelhardt & Klemme; 1982).

Similar findings have been made with several other Gram-negative bacteria (see Table 7. 1.) but the GS enzymes from Gram-positive bacteria have been shown not to be controlled by the covalent binding of adenylyl groups to the enzyme subunits although the general structure is the same (Deuel et al., 1970).

Table 7. 1. shows the known pH and temperature optima for the GS enzyme extracted from a number of Gram-negative bacteria which have been shown to be controlled by adenylylation.

Table 7. 1.

The published iso-active- and optimum-pH values for the GS from a number of Gram-negative bacteria.

ORGANISM		iso-pH	opt-pH	REFERENCE
T.ferrooxidans	NF	7.71	6.5	
	NH_4+	7.71	6.5	Barros et al., 1986.
K.aerogenes	NF	7.5	8.0	
	NH_4+	7.5	6.95	Bender et al., 1977.
M.ivanovi		NR	8.0	Bhatnagar et al., 1986.
V.alginolyticus	NF & NH_4+	none	7.9	Bodasing et al., 1985.
R.sphaeroides		6.95	NR	Engelhardt & Klemme; 1982.
S.typhimurium	NF	7.57	8.1	
	NH_4+	7.57	6.9	Janson et al., 1984.
E.coli	NF	7.15	6.9	
	NH_4+	7.15	8.0	Stadtman & Ginsberg, 1974.

Key: NR= not reported. NH_4+ = ammonia shocked, usually fully adenylylated. NF = grown in nitrogen limiting conditions, usually fully deadenylylated.

7. 2. 2. GAMMA-GLUTAMYL TRANSFERASE ASSAY.

The GGT assay reagent was prepared daily according to the method of Bender *et al.* (1977), and contained (final molarity): 2.25 ml of 1.0 M Imidazole-HCl pH 7.15 (135 mM), 0.37 ml of 800 mM hydroxyamine-HCL (18 mM), 0.045 ml of 0.1 M $MnCl_2$ (0.27 mM), 1.5 ml of 0.28 M potassium arsenate (25 mM), and 0.15 ml of 40 mM sodium ADP pH 7.0, and 7.53 ml of distilled water. If required a volume of 1.5 ml of 1 mg ml^{-1} CTAB was added or an equivalent amount of distilled water. The pH of the reagent was adjusted using 1 M KOH or 1 M HCL as required. Blank reagent was prepared by omitting potassium arsenate and ADP.

Where modifiers of the enzyme were to be added to the assay, water (1.5 ml) was omitted from the reagent and a solution (0.05 ml) containing the required substance was added to the assay. The final molar concentration in the assay of these substances is reported.

For the assay, sample (0.05 ml) was mixed with 0.4 ml of the reagent, equilibrated for 5 min at the required incubation temperature, and the reaction initiated by the addition of 0.05 ml of 0.2 M L-glutamine (20 mM). The reaction was terminated, typically after 15 min, by the addition of 1.0 ml "stop mix". This contained (g l^{-1}), $FeCl_3.6H_2O$ (55), trichloracetic acid (20), and concentrated HCl (21 ml).

The samples were centrifuged to remove precipitated protein and the absorbance at 540 nm measured in a Beckman DU-8 spectrophotometer. Three readings were taken of each sample and averaged. Samples were typically assayed in duplicate and averaged when standard errors from the mean were not greater than 10%.

The A_{540} was used to extrapolate the quantity of end product from a standard curve of glutamylhydroxamate, prepared under identical conditions, where 1 µmol of glutamylhydroxamate gave an A_{540} of 0.525. One unit of GS activity is defined as the amount of glutamylhydroxamate formed per min per mg of enzyme protein.

7. 2. 3. FORWARD SYNTHETIC TRANSFERASE ASSAY.

The FT assay reagent was prepared daily according to the method of Bender *et al.* (1977), and contained (final molarity): 2.0 ml of 1.0 M Imidazole-HCl pH 7.15 (94 mM), 1.25 ml of 800 mM hydroxyamine-HCL (47 mM), 0.040 ml of 0.1 M $MgCl_2$ (56 mM), 4.2 ml of 0.85 M monosodium L-glutamate (168 mM), and 7.2 ml of distilled water. If required a volume of 2.0 ml of 1 mg ml^{-1} CTAB was added or an equivalent amount of distilled water. The pH of the reagent was adjusted using 1 M KOH or 1 M HCL as required.

Where modifiers of the enzyme were to be added to the assay, a volume of 0.025 ml of sample was used and a solution (0.025 ml) containing the required substance was added to the assay.

The final molar concentration in the assay of these substances was reported.

For the assay, sample (0.05 ml) was mixed with 0.4 ml of the reagent, equilibrated for 5 min at the required incubation temperature, and the reaction initiated by the addition of 0.05 ml of 0.2 M ATP pH 7.7 (20 mM). The reaction was terminated, typically after 15 min, by the addition of 1.0 ml "stop mix". This contained ($g\ l^{-1}$), $FeCl_3.6H_2O$ (55), trichloracetic acid (20), and HCl (21 ml).

The samples were centrifuged to remove precipitated protein and the absorbance at 540 nm measured in a Beckman DU-8 spectrophotometer. Three readings were taken of each sample and averaged. Samples were typically assayed in duplicate and averaged when standard errors from the mean were not greater than 10%.

The A_{540} was used to extrapolate the quantity of end product from a standard curve of glutamylhydroxamate, prepared under identical conditions, where 1 µmol of glutamylhydroxamate gave an A_{540} of 0.510. One unit of GS activity is defined as the amount of glutamylhydroxamate formed per min per mg of enzyme protein.

7. 2. 4. SNAKE VENOM PHOSPHODIESTERASE TREATMENT OF GS.

Samples of GS were incubated at 37°C with 0.5 mg ml^{-1} of snake venom phosphodiesterase I (Sigma Chemicals), sampled at various time intervals and immediately frozen at -70°C. The samples were then assayed at the same time in the presence and absence of 10 mM Mg^{2+} for the E.coli enzyme or the presence and absence of 0.25 mM added Mn^{2+} for the putative B.fragilis enzyme.

ALKALINE PHOSPHATASE TREATMENT OF GS.

Alkaline phosphatase (AP) from calf intestine (Boehringer Mannheim) was diluted to 40 µg ml^{-1} in 0.1 M tris-HCL buffer at pH 7.9. This produced 0.738 A_{450} units of activity when tested as described in chapter 8.
Samples of crude GS (50 µl) were incubated with AP (25 µl) for 15 min at room temperature. The GGT assay reagent was then added to the mixture and the GS activity determined as µMol glutamylhydroxamate formed per min, per mg protein.

SECTION 3. RESULTS

7. 3. 1. EXTRACTS OF NF AND N+ CELLS.

E.coli YMC-10 $GlnA^+$ cells yielded extracts which contained 0.76 units (NF) and 1.004 units (N+) of GS specific activity by the GGT assay at the iso-activity point of pH 7.1.

E.coli YMC-11 (pJS139) $GlnA^+$ cells yielded extracts which contained 3.03 units (NF) and 1.67 units (N+) of GS specific activity by the GGT assay under optimal conditions. The FT assay for the same samples gave values of 1.054 units (NF) and 0.82 units (N+) of specific activity.

Aliquots of the enzyme extracts, stored at -70°C were stable for at least 6 months, and showed no change in activity in the GGT and FT assays .

7. 3. 2. pH OPTIMUM FOR THE CLONED GS ENZYME.

Cloned GS enzyme showed no real difference in pH optimum between extracts prepared from ammonia shocked and nitrogen starved cells. The NF extracts had an optimum of pH 6.5 to pH 7.0, while the N+ extracts had a lower specific activity and an apparent optimum of between pH 6.5 to pH 7.0 (Fig.7. 1.). The assays of the cloned GS which are reported here were carried out at pH 7.0.

7. 3. 3. OPTIMUM TEMPERATURE FOR THE CLONED GS ASSAY.

The optimum temperature for the assay of the cloned GS by the GGT assay was 45°C (Fig.7. 2). A temperature of 45°C was used for all other assays of this enzyme reported here. A temperature of 37°C was used for assays of GS extracted from E.coli YMC-10.

7. 3. 4. EFFECT OF FEED-BACK INHIBITORS ON THE CLONED GS.

Potential feed-back modification of GS activity by end products of glutamine metabolism was tested with a range of amino acids and amino compounds. All the amino containing compounds which were tested, exerted an inhibitory effect on the cloned GS in the GGT assay (Table 7. 2) with the exception of glutamine, a substrate in the assay. L-Methionine-DL-sulphoximine, a glutamate analog which non-competitively inhibits E.coli GS (Bhatnagar et al., 1986), inhibited the cloned GS from NF and N+ extracts by more than 90% both in the GGT and FT assays.

7. 3. 5. EFFECT OF ADDED GLUTAMINE IN THE GGT ASSAY.

Increased quantities of glutamine added to the GGT assay of NF cloned GS resulted in a marked increase in glutamyl-hydroxamate formation between 10 mM and 20 mM. The formation of glutamylhydroxamate virtually plateaued above 20 mM and further addition of glutamine caused only a small increase (Fig.7. 3). Glutamine (30 mM) was used as a substrate in the GGT assays reported here.

7. 3. 6.

EFFECT OF Mn^{2+} AND Mg^{2+} ON THE GGT ASSAY:

Addition of Mn^{2+} and Mg^{2+} to NF and N+ extracts of E.coli showed the effects reported by Stadtman & Ginsberg (1974). Increased concentrations of Mg^{2+} caused an increase in GS GGT activity of extracts from NF cells, while extracts from N+ cells were inhibited. Extracts of NF and N+ cells were similarly inhibited by concentrations of Mn^{2+} above 1.0 mM (Fig. 7. 4C & D).

In contrast, assays of extracts of the cloned GS with increasing quantities of Mg^{2+}, showed that both NF and N+ extracts were equally inhibited by increasing concentrations of this cation (Fig.7. 4A.).

Addition of Mn^{2+} indicated that low (0.25 mM) concentrations caused marked (100%) stimulation of the N+ extract while 0.5 mM Mn^{2+} had approximately the same activity as the assay without added Mn^{2+}. Concentrations of Mn^{2+} above 0.5 mM resulted in inhibition of GS activity. Concentrations of Mn^{2+} up to 0.5 mM had no effect on the activity of GS from NF cells, but above 0.5 mM Mn^{2+} the GS activity was inhibited (Fig.7. 4B.).

The GS activity of samples was assayed in the GGT assay containing 0.27mM $MnCl_2$, as described by Bender et al., (1977).

7. 3. 7. EFFECT OF PHOSPHODIESTERASE ON CLONED GS:

The extracts of the E.coli GS extracted from N+ cells showed the reported stimulation (Stadtman & Ginsberg, 1974) following diesterase treatment when assayed in the presence of 20mM added Mg^{2+} (Fig.7. 5E and 5F). The NF extracts (unadenylylated) were unaffected by the phosphodiesterase I treatment when assayed under these conditions.

As the addition of Mg^{2+} to assays of the cloned GS did not result in enhanced activity of NF extracts relative to N+ extracts (Fig. 7. 4A) it was decided that no point was served by assaying phosphodiesterase I treated GS samples in the presence of this cation. However as a difference in response between N+ and NF extracts was noted after addition of 0.25 mM Mn^{2+} to the assay (Fig. 7. 4B), samples of these extracts during treatment with phosphodiesterase I were assayed in the presence of 0.25 mM Mn^{2+}.

GGT assay of samples of the cloned GS following phosphodiesterase I treatment, with and without added Mn^{2+} showed that the GS enzyme activity from both NF and N+ cells was inhibited by the phosphodiesterase at 37°C. The addition of Mn^{2+} to this assay did not affect the inactivation (Fig.7. 5A-5D). Addition of Mg^{2+} (10 mM) to the diesterase treated, cloned GS samples did not change this result (data not shown).

As the specific activity of the N+ extract was reduced by the phosphodiesterase I treatment, the absolute difference between samples assayed in the presence and absence of Mn^{2+} was reduced (Fig. 7. 6.).

7. 3. 8. TREATMENT OF CLONED GS WITH ALKALINE PHOSPHATASE.

Treatment of preparations of cloned B.fragilis GS with AP for 15 min did not result in loss of activity in the GGT assay. Preparations of GS from E.coli YMC-10 NF and N+ cells were similarly unaffected by this treatment.

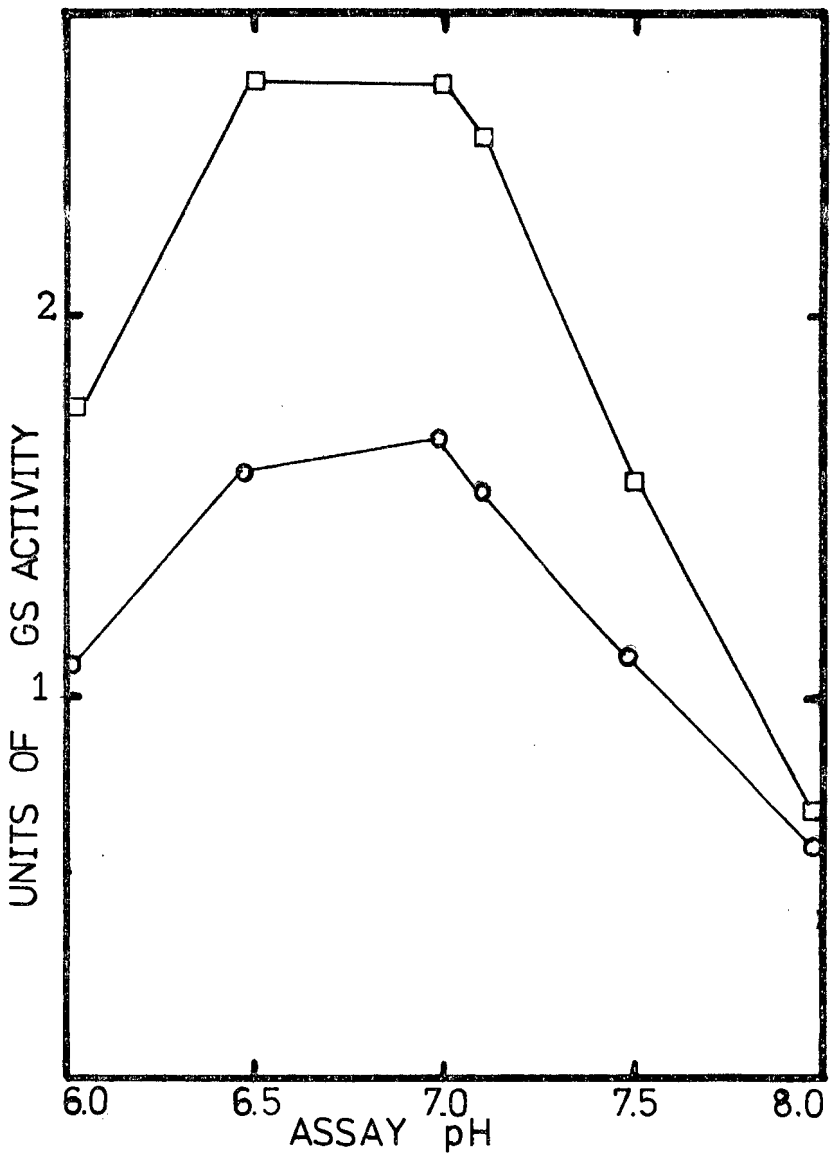


Fig. 7. 1. ACTIVITY OF THE CLONED GS AT VARIOUS pH VALUES
GS extracted from cells grown under nitrogen limiting conditions (□ - □), or following ammonia shock (o - o) was assayed for GGT activity at various pH values. Activity is expressed as μmol glutamylhydroxamate formed per min, per mg protein.

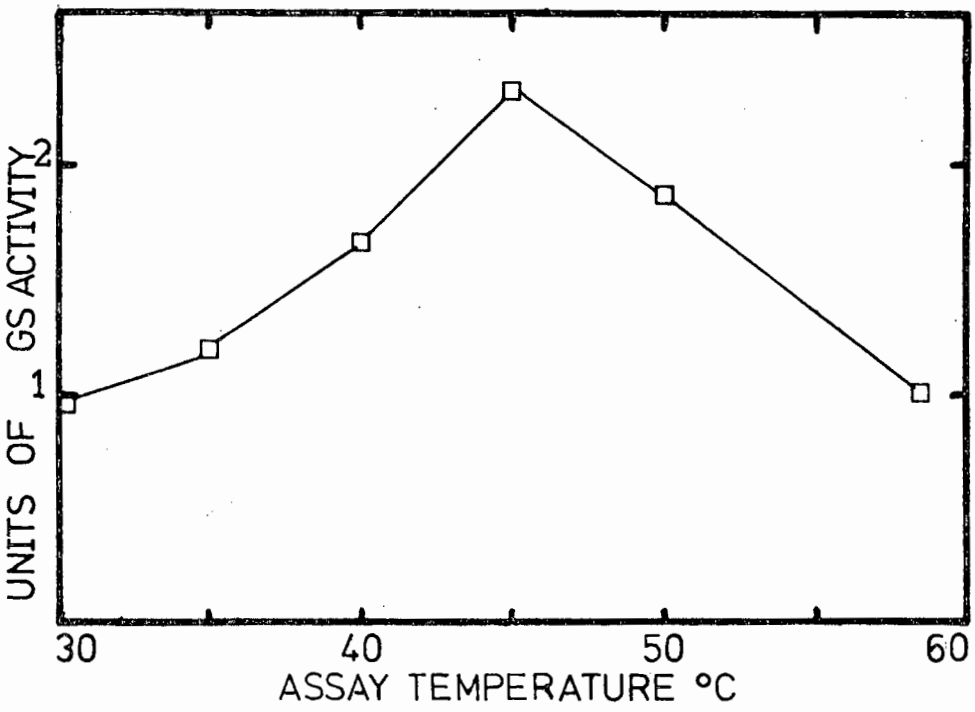


Fig. 7. 2.
OPTIMUM TEMPERATURE FOR THE ACTIVITY OF THE CLONED GS
GS extracted from E.coli YMC-11 (pJS139) was assayed at different temperatures by the GGT assay. Activity is expressed as µmol glutamyl-hydroxamate formed per min, per mg protein.

TABLE 7. 2.

EFFECT OF FEEDBACK MODIFIERS ON THE ACTIVITY OF THE CLONED GS

GS extracted from E.coli YMC-11 (pJS139) was tested by the GGT assay in the presence of the modifiers. The final concentration of the modifier in the assay is shown in brackets. The residual GS activity is expressed as a percentage of the activity (µmol glutamylhydroxamate per min, per mg protein) of the extract to which a similar quantity of distilled water had been added. The reported values are the mean of two assays which did not vary more than 10%.

FEEDBACK MODIFIER	PERCENTAGE RESIDUAL GS ACTIVITY
AMP (20mM)	104.5
L-PROLINE (50mM)	88.8
L-ISO-LEUCINE (50 mM)	85.6
$(NH_4)_2SO_4$ (50 mM)	81.6
L-GLYCINE (50 mM)	74.4
L-GLUTAMIC ACID (50 mM)	70.4
L-ALANINE (50 mM)	68.2
L-HISTIDINE (50 mM)	46.2
L-ARGININE (50 mM)	38.6
L-METHIONINE-DL-SULPHOXIMINE (0.1 mM)	8.0

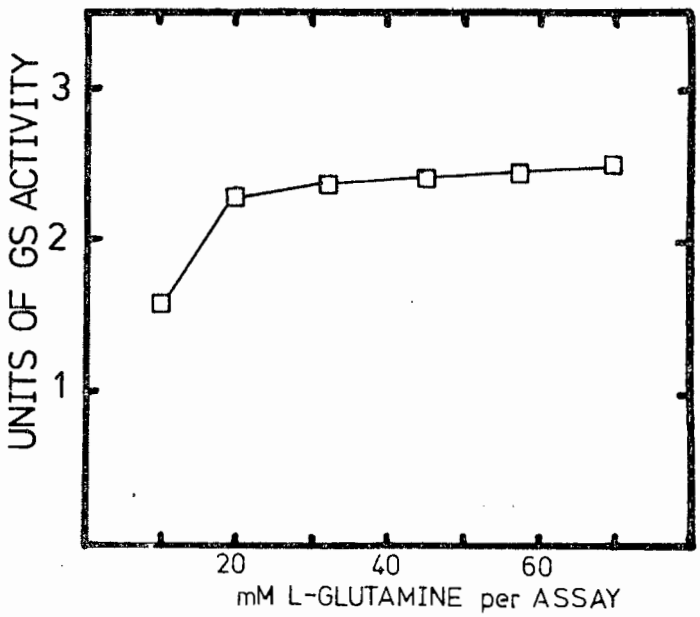


Fig. 7. 3. EFFECT OF L-GLUTAMINE CONCENTRATION ON THE CLONED GS ACTIVITY

GS extracted from E.coli YMC-11 (pJS139) was tested in the GGT assay with increasing concentrations of L-glutamine. The quantity used in the standard assay is 30 mM. GS activity is expressed as µmol glutamyl-hydroxamate formed per min, per mg protein.

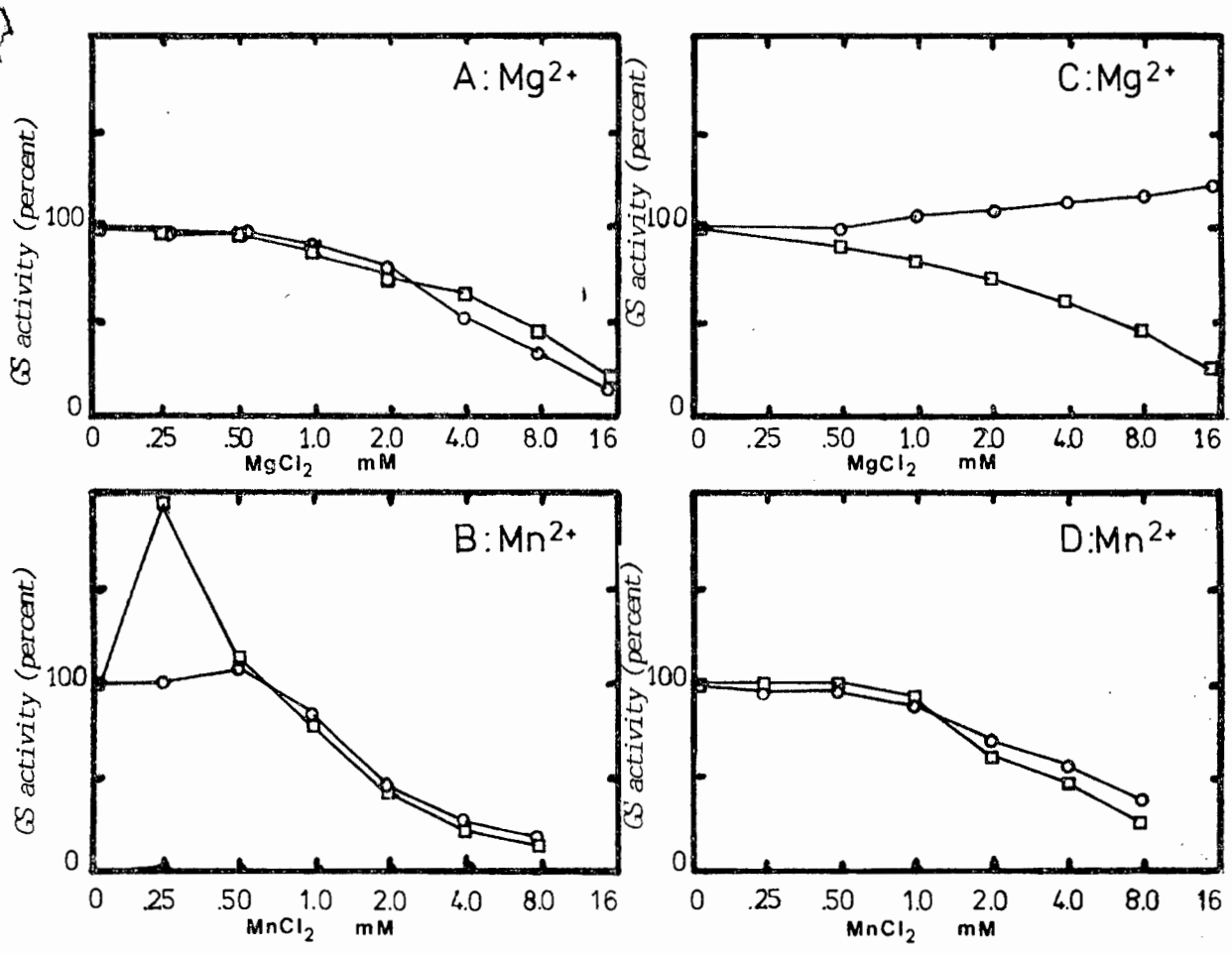


Figure 7. 4.
THE EFFECT OF DIVALENT CATIONS ON THE ACTIVITY OF THE CLONED GS

GS extracted from E.coli YMC-11 (pJS139) was assayed in the presence of increasing concentrations of, A: Mg^{2+}, and B: Mn^{2+}.

GS extracted from E.coli YMC-10 was assayed in the presence of, C: Mg^{2+}, and D: Mn^{2+}.

The results are expressed as a percentage of the standard GGT assay result with no added divalent cations (µmol glutamylhydroxamate formed per min, per mg protein) for each preparation.

Symbols: GS from N+ cells (-).
GS from NF cells (o - o).

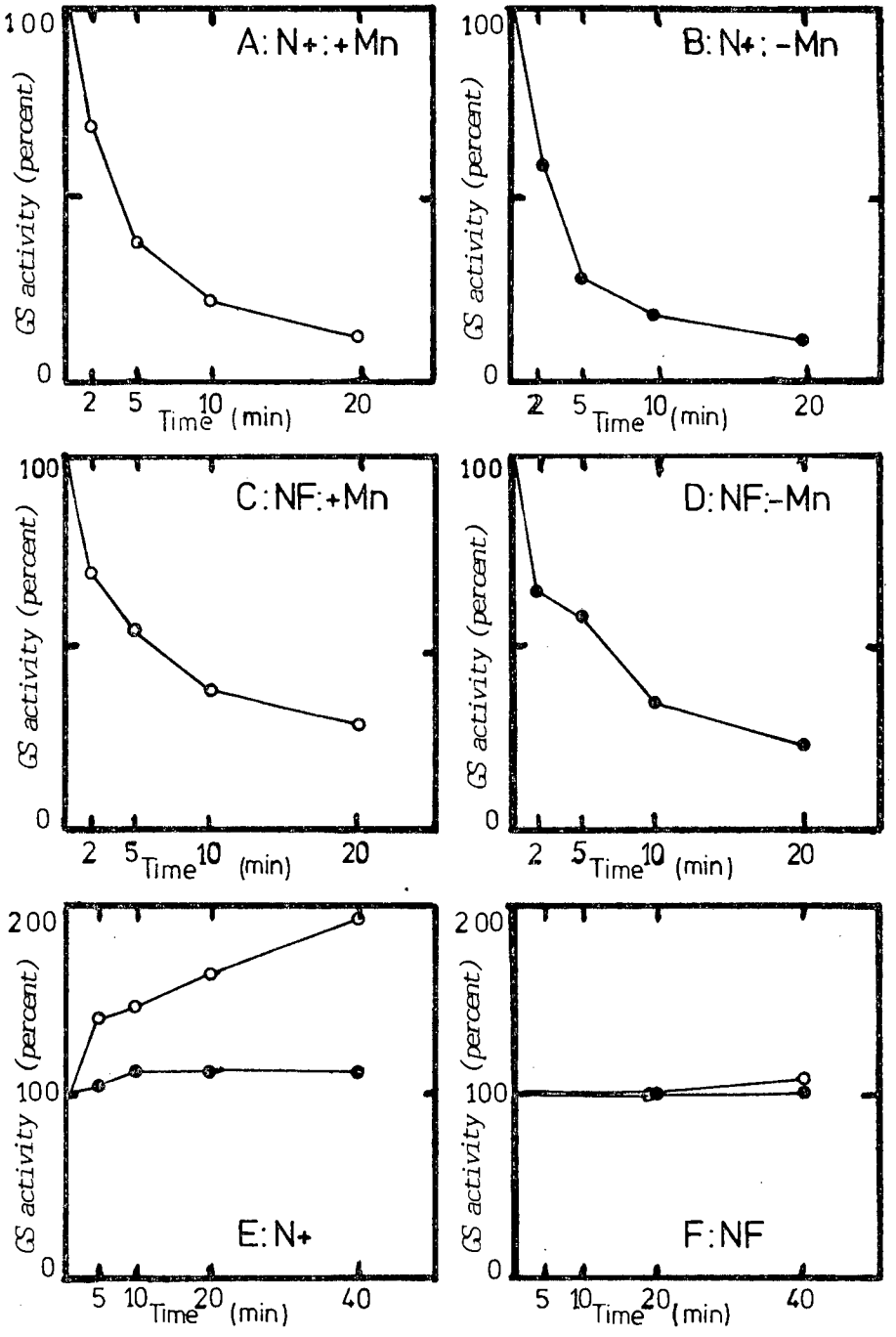


Fig. 7. 5. EFFECT OF PHOSPHODIESTERASE ON GS ACTIVITY
Snake venom Phosphodiesterase I (0.5 mg/ml) was incubated with GS extracts and samples assayed by the GGT assay at the indicated time intervals.

A & B: GS from N+ E.coli YMC-11 (pJS139), assayed with (A) and without (B), 0.25 mM $MnCl_2$.

C & D: GS from NF E.coli YMC-11 (pJS139), assayed with (C) and without (D), 0.25 mM $MnCl_2$.

E & F: GS extracted from N+ (E) and NF (F), E.coli YMC-10, assayed with (o - o) and without (● - ●) 20 mM $MgCl_2$.

Results are expressed as a percentage of the units of activity of the starting material (µmol/min/mg).

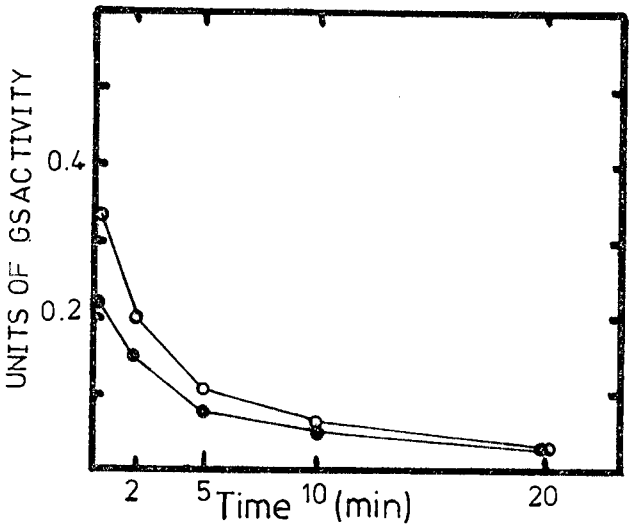


Fig. 7. 6.

EFFECT OF $MnCl_2$ ON PHOSPHODIESTERASE TREATED CLONED GS.

GS treated with phosphodiesterase I (0.5 mg ml^{-1}) for the time intervals shown was assayed for activity by the GGT assay, in the presence (o - o), and the absence (• - •) of added 0.25 mM $MnCl_2$. Enzyme units of activity are expressed as µmol glutamylhydroxamate formed per min, per mg protein.

SECTION 4. DISCUSSION

Extracts of E.coli YMC-11 (pJS139) cells grown under nitrogen limiting conditions contained more active GS than extracts from ammonia shocked cells (3.03 units and 1.67 units respectively). Due to the high activity of the NF extract, it was necessary to dilute this in C buffer to obtain reasonably comparable readings to the N+ extract in the subsequent tests. As the cultures were similar in all other respects, it is probable that the enzyme from the ammonia shocked cells had been inhibited in some way.

With the E.coli GS enzyme this inhibition is due to adenylylation of the enzyme. This results in changes to the behaviour of the enzyme in the GGT assay, in particular a change in the activity profile of the enzyme over a range of pH values (Bender et al., 1977). This change was not observed with the cloned GS, in particular there was no apparent cross-over of the curves of activity and the apparent optimum was the same for both extracts. Thus it was not possible to determine an iso-activity point for the cloned GS.

This apparent lack of a GS iso-activity point has also been reported for R.palustris, Chloroflexus aurantiacus and V.alginolyticus (Alef & Zumft; 1981, Kaulen & Klemme; 1983, and Bodasing et al.; 1985).

The optimum temperature for the activity of the cloned GS was determined to be 45°C. Temperature optima for most other bacterial GS enzymes have not been reported and 37°C is commonly used.

The activity of the cloned B.fragilis GS is sensitive to inhibition by amino acids. The degree of inhibition varied with the amino acid and ranged from approximately 10% to 60%. The glutamate analog L-methionine-DL-sulphoximine caused more than 90% inhibition of the cloned GS. Glutamine itself, stimulated the assay. These results are similar to those reported for E.coli, K.aerogenes, R.japonicum, V.alginolyticus and M.ivanovi (Woolfolk & Stadtman; 1967, Bender et al.; 1977, Bhandari et al.; 1983, Bodasing et al.; 1985, Bhatnagar et al.; 1986). However lack of inhibition of a cloned T.ferrooxidans GS by amino acids has been reported by Barros et al., (1986).

In contrast however to the reported results from the above bacteria, the activity of the cloned B.fragilis GS was not inhibited by the addition of AMP to the GGT assay.

The addition of divalent cations to the GGT assay of the cloned GS produced results which were not the same as the reported findings for the majority of Gram-negative GS enzymes. Mg^{2+} did not stimulate the GS extracted from nitrogen limited cells and both NF and N+ GS extracts were inhibited by Mg^{2+} to much the same extent.

The addition of Mn^{2+} in small quantities (0.25 mM) stimulated the cloned B.fragilis GS extracted from ammonia shocked cells, while GS extracted from cells grown under nitrogen limiting conditions was not stimulated. Further addition of Mn^{2+} however resulted in the inhibition of both the NF and N+ GS extracts. The significance of this finding was difficult to assess, and may have been an artifact of the extraction process, although it proved to be repeatable. The GS from another anaerobe, C.acetobutilicum, has been shown to be very sensitive to the concentration of Mg^{2+} (Usdin et al. 1986).

Phosphodiesterase I treatment of the extracts of the cloned B.fragilis GS caused the inhibition of enzyme activity in both NF and N+ extracts, and this inhibition was not affected by addition of either Mn^{2} or Mg^{2+} to the assay. Treatment of the extracts with AP did not however cause a reduction of GS activity in the GGT assay.

This unique finding may suggest that the enzyme is most active in a phosphorylated state, not necessarily adenylylated, and that this state is not changed by the growth conditions of the cells with regard to available nitrogen. However the extracted GS enzyme does exhibit differences between NF and N+ cells with regard to a requirement for Mn^{2+}.

CHAPTER 8

GLUTAMINE SYNTHETASE IN B.fragilis.

SUMMARY.

It has proved impossible to demonstrate substantial amounts of GS activity in B.fragilis BF-1, using either the GGT or forward transferase assay. Fresh extracts of cells prepared from nitrogen limited cultures showed traces of activity in the GGT and FT assay but this was transient and was rapidly lost. Using antiserum raised to the purified cloned GS, the presence of an immunologically related polypeptide in extracts of B.fragilis BF-1 was demonstrated by "western blotting" after SDS-PAGE. This polypeptide had the same M_r as the subunit of the cloned GS and its production was induced by growth of the B.fragilis strain in nitrogen limiting medium.

An inhibitor of the cloned GS was found to exist in extracts of B.fragilis BF-1 cells. This inhibitor was specific for the cloned GS and had only limited effects on crude extracts of GS from E.coli YMC-10 . This probably explains the difficulty in demonstrating GS activity in B.fragilis extracts.

SECTION 1. INTRODUCTION

GS FROM ANAEROBIC ORGANISMS:

Yamamoto et al. (1984) investigated the ammonia assimilation of B.fragilis grown in media containing various amounts of available nitrogen. They prepared extracts from cells grown in batch culture and from continuous cultures where available nitrogen was a growth limiting factor. They found that the major means of nitrogen assimilation was by the NADPH- and NADH-linked glutamate dehydrogenase pathway. Using glutamate dependent ATP hydrolysis, low levels of GS were detected in cell extracts, particularly from cells grown under nitrogen limiting conditions, but this enzyme was unstable and lost activity on storage. Although they could show that the GS from cells grown in the presence of L-methionine-DL-sulphoximine was 85% inhibited, the growth of these cells was not affected by the inhibitor. They could detect no activity in the GGT or FT assays of Bender et al. (1977).

GS activity has been reported in the rumen anaerobe Bacteroides amylophilus (Jenkinson et al., 1979) grown in continuous culture with limited ammonia. This enzyme activity was inhibited by the addition of ammonium ions, but from the responses of the enzyme to divalent cations in in vitro assays, this was probably not due to control by adenylylation. They suggested that the major means of ammonia incorporation into these cells is by the glutamate dehydrogenase pathway.

Multiple forms of GS have been found in Rhizobium, one of which is similar in character to that found in E.coli while another is heat-labile and not affected by adenylylation, but is repressed by the presence of ammonia. It appears to function during anaerobic growth and is essential for nodulation and the production of nitrogenase (Magasanik, 1982). Carlson and Chelm (1986) report that this form of GS (GSII) from Bradyrhizobium japonicum is similar in immunological cross-reactivity, structure and amino acid sequence to the plant GS enzyme, and suggest that this enzyme may have been transferred to the bacterium from a eukaryotic source.

Evestigneeva & Kaush (1983) report the presence of a third GS from Rhizobium lupini bacteroids.

The presence of an ATP-dependent GS from Selenomonas ruminantium has been reported which shows no evidence of control by adenylylation and is not active in the GGT assay (Smith et al., 1980). Succinovibrio dextrinosolvens has been reported to have a GS which has properties similar to those of the E.coli enzyme (Patterson & Hespell 1985).

GS from Gram-positive anaerobic organisms has properties which resemble GS from aerobic Gram-positive organisms rather than GS of anaerobic Gram-negative organisms.

Cloned GS from C.acetobutilicum showed no evidence for control by adenylylation/deadenylylation and was very sensitive to the concentraion of Mg^{2+} ions in the GGT assay (Usdin et al., 1986).

The GS of C.pasterurianum also shows no evidence of control by adenylylation and a stong requirement for at least 0.1 mM $MgCl_2$ in the GGT assay for optimal activity (Krishnan et al., 1986). The unique feature of this GS enzyme is the reported structure which consists of 20 identical subunits of M_r 50 000.

In order to detect the possible expression of the cloned GS gene in B.fragilis BF-1, the source of the cloned DNA, the immunological technique of "western blotting" was employed. Proteins from whole-cell lysates were separated by SDS-PAGE gels and bound to nitrocellulose membranes following electrophoretic transfer, as described by Towbin et al. (1979). These polypeptides can be detected in situ by a suitably specific antiserum and visualized by autoradiographic or enzymically active second antibodies. This method can reliably and quantitatively detect 20 ng of protein (Dennis-Sykes et al., 1985).

SECTION 2. MATERIALS AND METHODS

8. 2. 1. BACTERIAL GROWTH CONDITIONS:

B.fragilis BF-1 was cultured in Bacteroides broth at 37°C (see Appendix 2) until late log phase (OD_{600}=1.8). An equal volume of sterile, anaerobic NaCl (0.15 M) buffered to pH 7.4 with 0.05 M phosphate containing 2 g l^{-1} glucose was added to the culture and incubation continued for 2 h. The cells were collected by centrifugation, washed with C buffer,and were resuspended in 1% of the culture volume of C buffer containing lysozyme (1 mg ml^{-1}). After 15 min at room temperature the cells were cooled on ice, and disrupted by ultrasonication. The cell debris was removed by centrifugation and the supernatant tested by the GGT and FT assays for GS activity. Cell extracts were also prepared from cells which had been washed twice with 1/5 the culture volume of 0.01 M tris-HCl (pH 7.9), and from cells which had been subjected to the sucrose/EDTA - distilled water osmotic shock technique described in chapter 2.

Cell extracts for western blotting were prepared from B.fragilis cells grown in the minimal medium of Varel & Bryant (1974) supplemented with 15 mM L-glutamine for repressed enzyme and without supplement for induced enzyme extracts. E.coli strains were grown in CSH minimal medium without $(NH_4)_2SO_4$, supplemented with 15 mM L-glutamine for repressed enzyme or with 15 mM L-glutamate for induced enzyme extracts.

8. 2. 2. IDENTIFICATION OF GS ACTIVITY:

Extracts of B.fragilis were assayed by the GGT and FT tests described in chapter 7. The standard assay was modified, in that 50 mM $MnCl_2$ was used in the assay reagent, and the tests were carried out at 45°C. As the activity of B.fragilis extracts was so low, it was felt that it might be due to some non-specific effect. In an attempt to identify true GS activity, the known inhibitor of GS in the GGT assay, L-methionine- DL-sulphoximine (0.1 mM) was added to the GGT and FT assay of fresh B.fragilis extracts. If the detected activity was due to GS it should be reduced by the inhibitor.

8. 2. 3. ACTIVITY OF DIALYSED B.fragilis EXTRACTS:

Samples of fresh extracts of B.fragilis were dialysed for 4 h, at 10 to 15°C, using 50000 M_r cut off membranes (Spectropore corp.), against 200 volumes of C buffer. The buffer was changed at hourly intervals. The dialysed samples were then tested for activity in the GGT assay.

8. 2. 4. INHIBITION OF CLONED GS BY BACTERIAL EXTRACTS:

The effect of dialysed and undialysed extracts (0.5 mg ml^{-1}) of B.fragilis GS which had lost their GS activity were tested against active GS from E.coli YMC-10 and E.coli YMC-11 (pJS139). Similar extracts from E.coli YMC-11 GlnA$^-$ cells were also tested against the active GS preparations.

8. 2. 5. QUALITATIVE TESTS OF THE BACTERIAL EXTRACTS FOR PHOSHPHATASE AND PROTEASE ACTIVITY:

Phosphatase activity: Solutions of p-Nitrophenyl phosphate (1.0 mg ml^{-1}) in 0.1 M phosphate buffer (pH 6.1), 0.1 M Tris-HCl buffer (pH 7.4), and 0.1 M Tris-HCl buffer (pH 8.5) were incubated at 37°C with samples of extracts from B.fragilis and E.coli cells. The A_{440} was determined after 30 min vs an individual blank for each sample. Alkaline phosphatase (calf intestine) (2 µg) and phosphodiesterase I (10 µg) were tested under the same conditions.

Protease activity: Azocasein (2% in Tris-HCl, pH 7.4) was incubated at 37°C with B.fragilis extracts which had had low GS activity and the amount of A_{440} material remaining after 10% TCA precipitation measured.

8. 2. 6. IMMUNOLOGICAL DETECTION OF GS IN B.fragilis:

Western blotting from SDS-PAGE gels onto nitrocellulose membranes was carried out by the method of Towbin et al. (1979), The membrane was blocked with 10mM Tris HCL (pH 7.4), 0.15 M NaCl, 2% (w/v) nonfat dried milk (Johnson et al., 1984) and 0.05% (v/v) Tween-20 overnight at room temperature. The antibody (described in chapter 6) binding and the development of bands using a goat anti-rabbit serum conjugated to horseradish peroxidase was carried out according to Rybicki & von Wechmar (1982), except that Tween-20 was used in place of NP-40.

8. 2. 7. PURIFICATION OF GS FROM B.fragilis EXTRACTS.

Fresh B.fragilis BF-1 extracts which showed slight GS activity were subjected to the same technique of PEG purification as had been employed with the cloned GS extracted from E.coli in chapter 6. Samples from the various stages were tested for activity in the GGT assay.

Fresh B.fragilis BF-1 extracts were also subjected to the centrifugation purification method of Streicher & Tyler (1980). The extract was centrifuged at 15 000 G for 30 min and the supernatant then centrifuged at 100 000 G for 60 min. The pellet was resuspended in 10% of the original volume (P1), and the supernatant (S1) recentrifuged at 100 000 G for 120 min. The supernatant (S2) was collected and the pellet (P2) resuspended in 10% of the original volume. These samples were then assayed by the GGT assay.

SECTION 3. RESULTS

8. 3. 1. EXTRACTION OF GS FROM B.fragilis CELLS.

Several extracts of B.fragilis cells contained GS activity of between 0.01 and 0.05 μM glutamylhydroxamate formed per min in the GGT assay (0.005 to 0.01 in the FT assay). The specific activity (μM/min/mg) was so low (0.001 to 0.005, and 0.001 to 0.002 respectively) as to be virtually insignificant and at the limit of detection for the assay. The activity of the samples in the GGT and FT assays was lost on storage of the samples, even at -70°C. The activity of the extracts was not influenced by the assay temperature as assay at 37°C and 45°C gave similar results.

L-methionine DL-sulphoximine (0.1 mM) completely inhibited the activity of a fresh B.fragilis GS extract in both the GGT and the FT assay. Extracts which formed 0.04 μM glutamylhydroxamate per min in the GGT assay, produced less than 0.005 μM/min in the presence of the inhibitor. This indicated that the slight activity was probably true GS activty. The GS activity of extracts of E.coli YMC-10 and E.coli YMC-11 (pJS139) was reduced by 90% by the inhibitor.

8. 3. 2. DIALYSIS OF B.fragilis GS EXTRACTS:

Samples of B.fragilis extracts which had been dialysed using 50000 M_r membranes lost their GS activity, while extracts of E.coli YMC-10 and YMC-11 (pJS139) did not lose their GS activity during dialysis (Table 8. 1A).

8. 3. 3. PURIFICATION OF GS FROM B.fragilis EXTRACTS.

Purification of the GS from B.fragilis BF-1 extracts, using the differential PEG precipitation technique which had proved successful with the cloned GS (chapter 6), failed to yield any active GS at any of the stages of purification subsequent to the addition of the 4% (w/v) PEG 6K.

Extracts prepared from washed or osmotically shocked B.fragilis cells did not show an improved yield of active GS (Table 8. 1B).

The centrifugation procedure for GS purification concentrated the activity into the first 100 000 G pellet (P1), but as the overall protein content was also increased, there was no overall purification of the GS enzyme (Table 8. 1C). No other fractions showed activity.

TABLE 8. 1.

GS ACTIVITY OF BACTERIAL EXTRACTS:

A: FOLLOWING DIALYSIS

Bacterial extracts were dialysed using 50 000 NMW cutoff membranes and the residual GS activity determined in the GGT assay.

Source of extract	GS activity (μM/min/mg)	
	before dialysis	after dialysis.
B.fragilis BF-1	0.01	0.001
E.coli YMC-10	0.79	0.74
E.coli YMC-11 (pJS139)	2.96	2.71

B: EXTRACTED AFTER WASHING

Extracts of B.fragilis BF-1 prepared as described in the text were tested for GS activity in the GGT assay, directly after preparation and after 16 h at 4°C.

Treatment before	GS activity (μM/min/ml)	
Extraction	fresh extract	after storage .
None	0.04	0.005
Two washes of tris-HCL	0.03	0
Sucrose-osmotic shock	0.02	0

C: FOLLOWING CONCENTRATION

Bacterial extracts were subjected to differential PEG precipitation, and centrifugation in an attempt to purify or concentrate the GS activity.

Sample	GS activity	
	(μM/min/ml	(μM/min/mg) .
B.fragilis BF-1 extract	0.047	0.005
PEG ppt 4%	0	0
PEG ppt 6%	0	0
P1 first 100 000 ppt	0.15	0.007
S1 first 100 000 spnt	0.04	0.004

8. 3. 4. PHOSPHATASE AND PROTEASE ACTIVITY OF GS EXTRACTS.

PHOSPHATASE ACTIVITY; Fresh B.fragilis whole cell extracts produced substantial phosphatase activity, while phosphodiesterase I, and an extract of E.coli YMC-11 produced very little in comparison (see Table 8. 2). Boiled B.fragilis extracts contained no phosphatase activity.

PROTEASE ACTIVITY: There was no detectable protease activity in the azocasein test with fresh and dialysed B.fragilis whole cell extracts.

Table 8. 2.

PHOSPHATASE ACTIVITY OF B.fragilis EXTRACTS.
Bacterial extracts (50µl) were incubated at 37°C with p-nitrophenyl phosphate (300µl) for 30 min, distilled water (1ml) was added, and the A_{440} determined.

Source of extract (µg/50ul)	Phosphatase activity (A_{440}) at pH 6.1	7.4	8.5
B.fragilis BF-1 (500)	0.01	0.78	1.35
E.coli YMC-11 (500)	0	0.01	0.04
Phosphodiesterase I (10)	0	0.03	0.02
Alkaline phosphatase (2)	not done	0.45	0.93
B.fragilis (boiled) (87)	0	0	0

8. 3. 5. INHIBITORY ACTIVITY OF B.fragilis EXTRACTS ON GS:

The inhibition of GS in the GGT assay by B.fragilis extracts is shown in Table 8. 3. Both the untreated and the dialysed extract inhibited the activity of the cloned GS by over 90%, while the E.coli N+ GS was only inhibited by 25% and 16% respectively. Addition of Mg^{2+} (20 mM) to the inhibited E.coli GS further reduced its activity, had the GS been deadenylylated by the B.fragilis extract, this addition should have enhanced the GS activity.

Boiled B.fragilis extract was still an inhibitor of the cloned B.fragilis GS even though it had lost its phosphatase activity. Following boiling the extract contained approximately 30% of the amount of protein of the original extract.

Whole-cell extracts of E.coli YMC-11 caused 80% inhibition of the GS activity of the cloned B.fragilis extracts, but less than 10% inhibition of E.coli GS extracts. As the active cloned GS can be purified from E.coli YMC-11 (pJS139) extracts, during which the enzyme becomes more active (Table 6. 2), the inhibition by YMC-11 extracts must be different from the inhibition by B.fragilis extracts which appears to be irreversible.

TABLE 8. 3.

INHIBITION OF GS BY B.fragilis EXTRACTS.

Extracts prepared from
A: E.coli YMC-11 (pJS139) and
B: E.coli YMC-10 which were active in the GGT assay were assayed in the presence of extracts of B.fragilis cells (BF), dialysed extracts (dial), or boiled extracts. Other inhibitors tested were an extract from E.coli YMC-11 cells, fresh Bacteroides broth, supernatant from a B.fragilis culture, and Alkaline phosphatase (AP).
Units of GS are expressed as μmol glutamylhydroxamate formed per min, per mg protein.

GS SAMPLE	INHIBITOR (mg/ml)	GS activity (μM/min/mg)	Residual activity %.
A			
Cloned GS	none	2.03	100
..	BF (10)	0.17	8.3
..	BF-dial (10)	0.17	8.3
..	BF boiled (2.7)	0.34	16.8
..	YMC-11 (13)	0.41	20.0
..	Fresh BF broth	1.85	91.0
..	Used BF broth (spnt)	1.93	95.5
..	AP (2.0 ug)	2.11	104.0
B			
E.coli GS	none	0.85	100
..	BF (10)	0.64	75
..	BF-dial (10)	0.72	84.3
..	BF-dial (10) + $MgCl_2$ (20 mM)	0.29	34.3
..	$MgCl_2$ (20 mM)	0.31	37.2
..	YMC-11 (13)	0.78	91.9
..	AP (1.0 ug)	0.83	98.8

8. 3. 6. IMMUNOLOGICAL DETECTION OF GS IN B.fragilis:

The western blot (Figure 8. 1.) shows a clear reaction between the antiserum to the cloned GS and a polypeptide in the whole cell lysates of E.coli YMC-11 (pJS139). This polypeptide is the same M_r as the subunit of the purified cloned GS and its quantity is increased in derepressed cells relative to the repressed cells.

Figure 8. 2. shows that the antiserum to the cloned GS did not react with whole cell lysates of E.coli YMC-11 or E.coli YMC-10. Further, it did not react with the E.coli GS sub unit, the presence of which was later revealed by reaction with another serum (raised against V.alginolyticus GS; Barros et al., 1986), known to react with E.coli GS. This antiserum also showed that there were no cross reacting GS polypeptides of M_r 50 000 in either of the YMC-11 extracts. In other blots, not shown here, this other anti-GS serum was shown to react weakly with the cloned GS. This second anti-serum also cross reacts with some other E.coli protein of about 52000 in all E.coli preparations. Extracts of E.coli YMC-11 GlnA$^-$ cells do not react with either anti-GS serum except for the 52000 cross reacting polypeptide.

Cell lysates prepared from B.fragilis react with the anti-serum to the cloned GS (Fig. 8. 3.) and the M_r of the polypeptide which reacts is the same as that of the cloned GS.

B.fragilis grown under nitrogen limiting conditions shows an enhanced reactive band even though the quantities of protein loaded onto the gel were equivalent. The antiserum reacted strongly with a polypeptide of the same M_r as the cloned GS in lysates of E.coli YMC-11 (pJS139). These lanes were heavily loaded and several cross reacting proteins are visualized in the E.coli extracts.

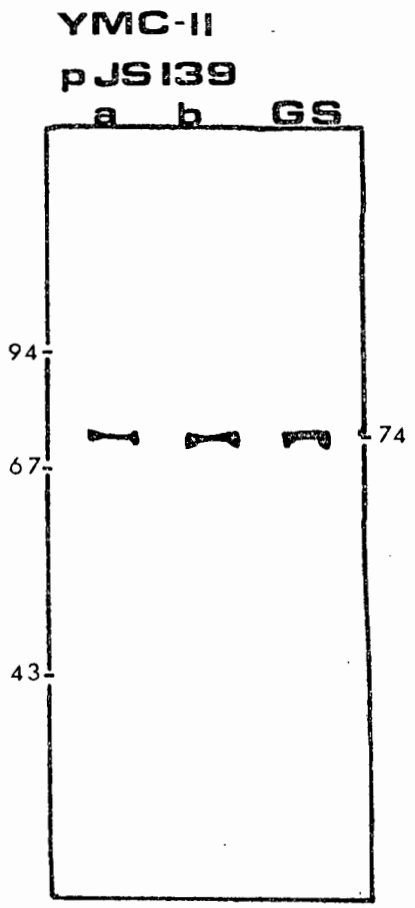


Fig. 8. 1. DETECTION OF GS IN E.coli YMC-11 (pJS139).

The western blot was carried out as described in the text from an 8% SDS-PAGE gel of (GS) purified cloned GS (1ug) and whole cell lysates of E.coli YMC-11 (pJS139), (a) grown in the presence of glutamine (repressed), and (b) grown in the absence of glutamine (derepressed), 10 ug each. The position of M_r markers are shown on the left (X 10^3) and the calculated M_r of the GS on the right (X 10^3). The antiserum described in chapter 6 was used diluted 1/80.

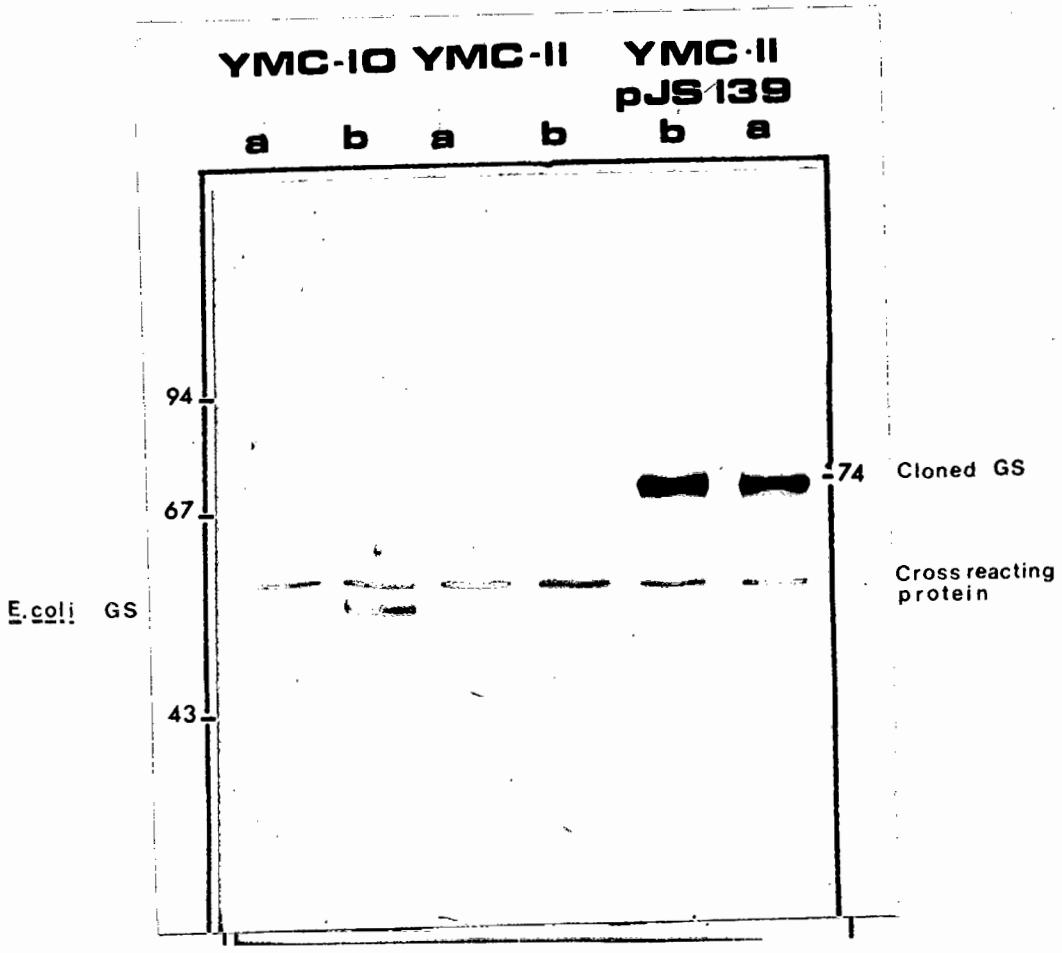


Fig. 8. 2.

WESTERN BLOT OF E.coli CELL LYSATES vs GS ANTISERA.

The western blot was carried out as described in the text from an 8% SDS-PAGE gel of E.coli whole cell lysates (10 ug each) and reacted initially with the anti-GS serum (chapter 6). In this reaction only the bands at 74 000 were visible. Subsequent reaction of this blot with an anti-serum which reacts with E.coli GS revealed the lower M_r bands. The cell lysates were prepared from cells grown (a) in the presence of glutamine (repressed) and (b) in the absence of glutamine (derepressed). The position of M_r markers are shown on the left (X 10^3). The second antiserum also reacted with a polypeptide of approximately 54 000 in all the E.coli extracts. This is believed to have no relationship to the GS polypeptide.

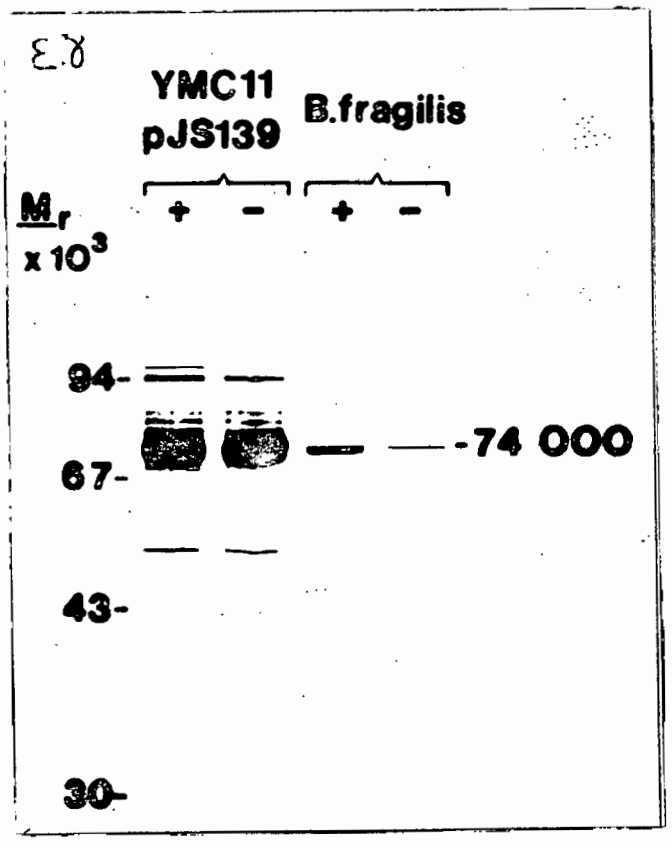


Fig. 8. 3. DETECTION OF GS POLYPEPTIDE IN B.fragilis.

The western blot was carried out as described in the text from an 8% SDS-PAGE gel of bacterial whole cell lysates (20 ug each) and reacted with the anti-GS serum described in chapter 6. The cell lysates were prepared from GS repressed cells (-), grown in the presence of glutamine and GS induced cells (+), grown in the absence of glutamine. The position of M_r markers are shown on the left ($X\ 10^3$). The polypeptide band from B.fragilis which reacts with the antiserum to the cloned GS is the same M_r as the polypeptide subunit of the cloned GS (74 000), and shows evidence of induction by growth of the cells on a nitrogen limiting medium. The lane containing the E.coli extracts are heavily loaded and show several E.coli polypeptides which cross react with the antiserum.

SECTION 4. DISCUSSION

It was possible to detect small amounts of GS activity in extracts of B.fragilis, but this activity was unstable and rapidly lost. That it was specific GS activity is suggested by the inhibition of activity in fresh B.fragilis lysates by the glutamate analog L-methionine-DL-sulphoximine. Techniques aimed at reducing extracellular cell bound material and periplasmic proteins, which might inhibit the intracellular GS did not succeed in enhancing either the recovery of the enzyme or the stability. Dialysis of extracts resulted in a total loss of activity, and it was not possible to purify active enzyme away from inhibitors using the differential PEG precipitation which had proved successful with the cloned B.fragilis GS in E.coli YMC-11 (pJS139).

The very sensitive technique of western blotting was used to show the presence in whole B.fragilis cell lysates of a polypeptide which reacted with antiserum raised to the cloned GS. This antiserum did not react with GS from E.coli. The B.fragilis polypeptide was the same M_r as that of the cloned GS, and the quantity of this polypetide was increased by growth in nitrogen limiting medium.

This indicates that:

1. It is the B.fragilis BF-1 GS which has been cloned into E.coli YMC-11 (pJS139).

2. The GS gene is expressed in B.fragilis and the translation product is the same as the cloned GS.

3. The GS may be functional in B.fragilis as the western blot shows induction of the B.fragilis GS in response to growth on nitrogen limiting medium.

4. The B.fragilis GS is only slightly related immunologically to the E.coli GS.

and 5. Since the enzyme is present in B.fragilis its detection may be hampered by the presence of an inhibitor or inactivator.

The presence of such an inhibitor was shown by the addition of B.fragilis cell extracts to assays of the cloned GS. Substantial inhibition of the enzyme occurred and this was more apparent in assays of the cloned GS than those of the E.coli GS (Table 8. 3).

The inhibition of the E.coli GS by the extract could have been due to the presence in the crude extract of feedback inhibitors such as amino acids which may have remained following dialysis. There was no deadenylylation of the E.coli GS enzyme as the activity in the presence of added Mg^{2+} was further depressed.

The extracts contained no protease activity measured by the azocasein method, but considerable amounts of phosphatase activity was present. This was noted during the purification of the bacteriocin in chapter 2.
The boiling of B.fragilis extracts resulted in a loss of phosphatase activity but the ability to inhibit the cloned GS was still present. This indicates that the inhibition is not caused by the action of an enzyme but by some other mechanism.

The following explanation for these findings is suggested. The GS produced in B.fragilis is intracellular, while the the inhibitor may be extra-cellular, perhaps a part of the periplasmic fraction. During the extraction process these, normally compartmentalized substances become mixed and the GS rapidly inactivated.

Further work to clarify this issue could include affinity chromatographic purification of GS from B.fragilis extracts using the antiserum to the clone GS, extraction of B.fragilis under different conditions, and analysis of B.fragilis extracts in an attempt to identify the inhibitor.

A B.fragilis GlnA$^-$ mutant is required before it will be possible to transform derivatives of the pJS139 plasmid into this organism. Until the GS from B.fragilis can be reliably estimated, it will be difficult to conduct further analysis of the cloned GS expression in B.fragilis.

SUMMARY AND CONCLUSIONS

The aim of this study was to investigate the molecular genetics of B.fragilis. The strains used had been studied with regard to their physiological responses to a variety of stress factors (reviewed by Woods & Jones, 1986), and an attempt was made to link these studies to the molecular biology of the organism.

The bacteriocin which was successfully isolated and purified was different from that previously reported for this strain. Although the reasons for this were sought, there was no clear explanation. The results of Mossie et al. (1979, 1980, 1981) may have been an artifact due to impurities in the purified bacteriocin, but this does not seem likely as their results were internally consistent. The reported in vivo inhibition of RNA synthesis was supported by the in vitro results, and further, the resistance to rifampicin was associated with resistance to the bacteriocin. The most plausible explanation is that there was a strain difference; either one of the strains was not BF-1, or BF-1 had changed by perhaps the loss of a plasmid, but this seems unlikely as no plasmid could be detected by Mossie et al. (1979).

Other workers have since reported the presence of both types of bacteriocins from their isolates of B.fragilis (Riley & Mee, 1985).

The indicator strain was continually in use by other workers so that a change in its nature would have been noted. However a cryptic plasmid was noted in this strain and investigated further.

The effect of the bacteriocin appears to be the lysis of growing sensitive bacteria, which may be caused by the inhibition of murein synthesis, or its hydrolysis, as with colicin M, which causes both (Schaller et al., 1982), or by some other unknown means. The Mr of the bacteriocin was smaller than any reported for Bacteroides, up until 1983, when most of this work was done. Three other bacteriocins of a similar size have been reported and two of these have been isolated from strains of Bacteroides.(Frick et al., 1981; Hayes et al., 1983; Austin-Prather & Booth, 1984).

The cryptic plasmid from the bacteriocin sensitive B.fragilis BF-2 strain was isolated and charaterised. Fragments of this plasmid were cloned into an E.coli vector and antibiotic resistance genes from a conjugal resistance transfer plasmid of Bacteroides which should function in B.fragilis were also inserted.

It was not possible to transform these hybrid plasmids into the strains of Bacteroides which were available, but this could be attempted when more competent strains are available.

A genetic library of the B.fragilis BF-1 strain was established using the positive selection vector pEcoR251. The available evidence suggests that the major part of the bacterial genome is represented in this library. Genes which complemented the auxotrophic mutations in E.coli were detectable as was a gene (or perhaps genes) which protected against DNA damaging agents.

The unequivocal expression of a gene for the synthesis of glutamine was detected. This enzyme had properties which differed from those of the E.coli enzyme.

The purified, cloned enzyme consisted of only six subunits, rather than the twelve reported for other bacteria, and the subunit size was substantially larger (M_r 75000) than that of other bacteria (50000 to 60000).

The control of the enzyme was different from that of the majority of other Gram-negative bacteria, particulary with regard to adenylylation.

Small amounts of a glutamine synthetase enzyme could be detected in B.fragilis, and an immunologically related polypeptide with the same M_r as the cloned enzyme was found in extracts of B.fragilis. These extracts contained a potent inactivator of glutamine synthetase activity.

APPENDIX. 1.

BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY.

STRAIN	RELEVANT GENOTYPE	SOURCE or REFERENCE.
BACTEROIDES		
B.fragilis BF-1	Wild type, bacteriocin^{+}	Mossie *et al.*,1979.
B.fragilis BF-2	pBFC1, bacteriocin indicator.	Mossie *et al.* 1979.
B.fragilis 4003	pBFTM10, *clin*^{r}.	Tally *et al.* 1979.
ESCHERICHIA		
E.coli K12 HB101	*pro*, *leu*, *recA*, *amp*^{s}, *strep*^{r}, *hsdS*$^{-}$(r^{-}, m^{-}), *glnA*$^{+}$, *ntrB*$^{+}$, *ntrC*$^{+}$.	Maniatis *et al.* 1982.
E.coli K12 CSR603	*recA*, *uvrA*, *phr-1*,	Sancar *et al.* 1979.
E.coli YMC-10	*glnA*$^{+}$, *ntrB*$^{+}$, *ntrC*$^{+}$, *Ap*^{S}	Backman *et al.*,1981
E.coli YMC-11	*glnA*$^{-}$, *ntrB*$^{-}$, *ntrC*$^{-}$, *Ap*^{S}	Backman *et al.*,1981.
E.coli ET8051	*glnA*$^{-}$, *ntrB*$^{-}$, *ntrC*$^{-}$, *Ap*^{S}	Tuli *et al.*,1982.

BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY.

continued.

STRAIN	RELEVANT GENOTYPE	SOURCE or REFERENCE.
PLASMIDS		
pBFC1	Cryptic plasmid	This study.
pBFTM10	$clin^r$, Tn4400,	Tally et al. 1979.
pBR325	amp^r, tet^r, cml^r,	Bolivar et al. 1977.
pEcoR251	Ap^r, EcoR1,	M.M.Zabeau, Plant genetic Systems, Ghent, Belgium.
pC_{I857}	Kan^r, Lambda P_R repressor(ts)	M.M.Zabeau, Plant genetic Systems, Ghent, Belgium.
pJS128a	tet^r, cml^r, (pBFC1-PstIa+b)	This study.
pJS128b	tet^r, cml^r, (pBFC1-PstIa)	This study.
pJS128c	tet^r, cml^r, (pBFC1-PstIb)	This study.
pJS128d	tet^r, cml^r, (pBFC1-PstIa+b)	This study.
pJS139	Ap^r, $glnA^+$,	This study
pJS139.1	Ap^r, $glnA^-$, (XhoI deletion)	This study
pJS139.2	Ap^r, $glnA^+$, (StuI deletion)	This study
pJS140z	tet^r, (pJS128a + pBFTM10-EcoRIb)	This study.
pJS140y	tet^r, (pJS128b + pBFTM10-EcoRIb)	This study.

APPENDIX 2.

FORMULATIONS OF BACTERIAL GROWTH MEDIA USED IN THIS STUDY.

1. Luria broth: (Davis et al., 1980).

Tryptone (Difco)	10.0g
Yeast extract (Difco)	5.0g
Sodium chloride	5.0g
Distilled water to	1000mℓ

The ingredients were dissolved, the pH adjusted to pH7.4 and autoclaved at 121°C for 15min.

For agar plates 15g of agar (Difco), and for "sloppy" overlay, 7.5g per litre was added.

2. Brain-heart infusion broth: (Mossie, 1979).

Brain heart infusion broth (Difco)	37.0g
Yeast extract (Difco)	5.0g
Sodium thioglycollate	1.1g
Sodium carbonate	4.0g
Resazurin	20mg
Distilled water to	1000mℓ

The ingredients were mixed and heated until the indicator was colourless. Hemin (5mg) and menadione (Sigma Chemical Co.) (0.5mg) were added, and CO_2 and H_2 bubbled through the medium to adjust the pH to 7.4. The medium was dispensed under the gas mixture and autoclaved at 121°C for 20min.

Brain-heart infusion agar plates:

These were prepared from Brain-heart infusion broth (Difco) according to the manufacturers directions. For standard agar plates, 15g/1 agar was added and for "sloppy" overlays for bacteriocin assay plates, 7.5g/1 of agar was added.

3. Bacteroides Broth: (Southern et al., 1984)

Tryptic soy broth (Difco)	24.0g
Yeast extract (Difco)	10.0g
glucose	1.0g
L-cysteine-hydrchloride	0.5g
Distilled water to	1000mℓ

The ingredients were dissolved, 10mg of resazurin (10mg) was added if required, and autoclaved at 121°C for 30min. The medium was cooled under a flow of N_2, H_2 and CO_2 (vols 80: 10: 20). Hemin (5mg) and menadione (0.5mg) was added from a filter sterilized stock solution.

4. CSH Minimal Medium: as used (Davis et al., 1980).

SALTS SOLUTION

Potassium dihydrogen phosphate	4.5 g
di Potassium hydrogen phosphate	10.5 g
Sodium citrate	0.47g
Ammonium sulphate	1.0 g
Magnesium sulphate . $7H_2O$	0.1 g
Distilled water to	100 mℓ

Dissolve in order, distribute in 10mℓ amounts and autoclave 121°C for 15 min.

BROTH BASE

Sodium chloride	5.0 g
Glucose	3.0 g
Distilled water to	900mℓ

Dissolve, distribute in 90mℓ amounts and autoclave 121°C for 15 min.

AGAR BASE

Sodium chloride	5.0 g
Glucose	3.0 g
Agar (Difco)	15.0 g
Distilled water to	900mℓ

Dissolve, distribute in 180mℓ amounts and autoclave 121°C for 15 min.

For use add one part of the salts solution to 9 parts of the base; the agar base must be melted and cooled to 50°C. Vitamin B_1 must be added from a sterile stock to lug/mℓ.

5. Bacteroides Minimal Medium: (Varel & Bryant, 1974).

MINERALS STOCK SOLUTION :

KH_2PO_4	18.0g
NaCl	18.0g
$CaCl_2.2H_2O$	0.53g
$MgCl_2.6H_2O$	0.4 g
$MnCl_2.8H_2O$	0.2 g
$CoCl_2.6H_2O$	0.02g
Distilled water	100mℓ.

STOCK SOLUTION II:

$FeSO_4.7H_2O$	4.0 g
Distilled water	100mℓ

GLUCOSE STOCK SOLUTION III:

Glucose	0.5 g
Distilled water	100mℓ

VITAMIN B_{12} STOCK SOLUTION IV:

Vitamine B_{12}	0.01g
Distilled water	100mℓ

Ten mℓ of the above stock was diluted into 100ml distilled water and filter sterilized.

HEMIN and MENADIONE STOCK SOLUTION:

Hemin (50 mg), NaOH 1M (1.0 mℓ); dissolve, add distilled water, make up to 100mℓ. Menadione (Vitamin K) (100 mg) + Ethanol 96%, (20 mℓ); Dissolve and store at 4°C.

For use add 1mℓ of menadione stock to 100mℓ of Hemin, filter sterilize and store at 4°C.

Preparation of MINIMAL MEDIUM

Mineral stock solution	25.0 mℓ
Solution II ($FeSO_4$)	5.0 mℓ
Resazurin	1mg
Ammonium sulphate	20mg
Sodium thioglycollate	550mg
Distilled water	395.0 mℓ

Autoclave 121°C for 20 min. Cool and add:

Glucose (10%) stock solution	25.0 mℓ
Vitamin B_{12} stock	2.5 mℓ
Hemin and Menadione stock	5.0 mℓ
Sodium carbonate (20%) stock	10.0 mℓ

The medium was aseptically dispensed into sterile Hungate tubes under CO_2 gas; or cooled in bulk under a stream of gas.

APPENDIX 3:

FORMULATIONS OF BUFFERS, STAINS AND SOLUTIONS USED IN THIS STUDY.

1. TE Buffer: (Davis et al., 1980).

Tris(hydroxymethyl)aminomethane 10mM

di-Sodium EDTA 1mM

HCl to adjust to pH 8.0.

This can be prepared as a 100 times concentrate, autoclaved at 121°C for 15 min, and diluted for use.

2. TMS Diluent:

Tris(hydroxymethyl)aminomethane - HCl pH 8.0 10mM

Mg_2SO_4 10mM

NaCl 150mM

Dissolve, distribute and autoclave 121°C for 15 min.

3.Aceto-orcein stain.

Orcein powder 5g

dissolve in 50%(w/v) Acetic acid and filter before use. Stain sample for 5min then decolourize with absolute alcohol.

3. Plasmid Extraction : (Ish-Horowitz & Burke, 1981)

Solution I:

Tris-HCl pH8.0	25mM
di-Sodium EDTA	10mM
Glucose	50mM

Sterilize by autoclaving at 121°C for 15min.

Solution II:

Sodium hydroxide	0.2N
SDS	1%(w/v)

This must be freshly prepared every week.

Solution III:

Potassium acetate	3M
Acetic acid	2M

or

Pot. hydroxide	13.44g

dissolve in approximately 40mℓ distilled water, adjust to pH7.0 with glacial acetic acid, then add 10mℓ of acetic acid, and make up to 80mℓ.

Cool on ice before use.

C BUFFER:

This was used for Sephacryl S-1000 and S-400 chromatography and for diluting GS samples.

10 mM imidazole-HCl pH 7.1

100 mM KCl

APPENDIX 4:

ELECTROPHORETIC TECHNIQUES USED IN THIS STUDY.

1. Electrophoresis of DNA in Agarose:

The methods are based on those of Helling et al., (1974).

Tris-acetate buffers were used for electrophoresis when the DNA fragments were to be transferred to a nitrocellulose membrane by Southern blotting for hybridization and autoradiography.

Tris-Borate buffers allow higher current densities and better resolution.

Agarose: HGT (FMC Corp.) or Biorad agarose were suspended at concentrations of 0.5 to 1.2%(w/v) in the buffer and dissolved by boiling. The agarose was cooled to about 55°C before the gel was poured. After the gel had set at room temperature it was cooled at 4°C for 15min before use.

Apparatus: Horizontal, flat bed, submarine electrophoresis was carried out in either a Hoefer HE99 apparatus or a locally made apparatus based on microtitre tray dimensions.

BUFFERS FOR AGAROSE GEL ELECTROPHORESIS:

TRIS-BORATE BUFFER:

Tris(hydroxymethyl)aminomethane	88mM
Boric acid	88mM
di-Sodium EDTA	1.25mM

pH8.0.

TRIS-ACTETATE BUFFER:

Tris(hydroxymethyl)aminomethane	40mM
Sodium actetate	20mM
di-Sodium EDTA	1.25mM

pH7.8.

ETHIDIUM BROMIDE STAIN:

A stock solution of 1mg/mℓ in TE was diluted 1/100 in the electrophoresis buffer for use.

3. SDS-polyacrylamide gel electrophoresis of Protein:

The method was a modification of the methods of Laemmli (1970) and O'Farrell (1975).

Apparatus: Pharmacia GE-2/4 LS was adapted for the use of thin (0.5mm) gels by the use of 2mm and 3mm glass plates, ABS plastic (0.5mm) spacers and combs. The plates were sealed with yellow electrical tape (3M Corp.) and a small quantity of 1% (w/v) agarose in lower gel buffer was run around the inside edges of the warmed (50°C) plates.

SOLUTIONS

SAMPLE LYSIS BUFFER:

Glycerol	10% (v/v)
2-Mercaptoethanol	5% (v/v)
Sodium dodecyl sulphate	2.3% (w/v)

in Tris-HCl (pH6.8) 0.0625M.

LOWER GEL BUFFER STOCK:

Sodium dodecyl sulphate	0.4% (w/v)

in Tris-HCl (pH8.8) 1.5M.

UPPER GEL BUFFER STOCK:

Sodium dodecyl sulphate	0.4% (w/v)

in Tris-HCl (pH 6.8) 0.5.

ACRYLAMIDE STOCK:

Acrylamide (Biorad)	29.2% (w/v)
NN-methylene bisacrylamide	0.8% (w/v)

in distilled Water, store 4°C.

ELECTROPHORESIS BUFFER:

Tris(hydroxymethyl)aminomethane	0.025M
Glycine	0.192M
Sodium dodecyl sulphate	0.1%(w/v)

FIXATIVE/DESTAINING SOLUTION:

Propan-2-ol	25.0% (v/v)

in 10%(v/v) Acetic acid.

COOMASSIE BLUE STAIN:

Coomassie brilliant blue R250	500mg
Fixative solution	500.0 mℓ

Dissolve stain in a little absolute alcohol, then add the Fixative solution and filter through Whatman GFA.

RESOLVING GEL MIX:

Quantities for two 150X150X0.5mm gels of 10.6%.

Acrylamide stock	12.0 mℓ
Lower gel buffer	8.2 mℓ
Distilled water	13.65mℓ

Mix, add:

TEMED (Biorad)	18uℓ
Ammonium persulphate (10%)	160uℓ

STACKING GEL MIX:

Acrylamide stock	2.0 mℓ
Upper gel buffer	3.0 mℓ
Distilled water	7.0 mℓ
TEMED (Biorad)	13uℓ
Ammonium persulphate (10%)	64uℓ.

4. Non-denaturing electrophoresis of Protein:

A 5-15% polyacrylamide gradient gel was prepared using 0.1M phospate buffer (pH8.25) with no SDS. The samples of purified bacteriocin were dissolved in TMS buffer and about 10ug was loaded in each lane. Electrophoresis was at 200V for 2h. The result is shown in Fig. 2. 6.

5. Pore-gradient (PG) electrophoresis of proteins:

A 4-30% polyacrylamide gradient gel was prepared in PG buffer (Pharmacia corp.) and polymerised with ammonium persulphate and TEMED. The same buffer was used for electrophoresis and the gel was pre-run at 70 V for 1 h before loading. Electrophoresis was for 7 h at 330 V, a total of 2310 Vh. The gel was fixed and stained with coomassie blue (see 3 above). Molecular weights were extrapolated from a graph of the mobility of each marker relative to the dye front (R_f).

APPENDIX 5

EXTRACTION AND PURIFICATION OF RIBONUCLEIC ACID POLYMERASE FROM B.FRAGILIS

The enzyme responsible for the transcription of DNA to RNA in bacteria has been extensively studied, especially in E.coli (reviewed by Kumar, 1981). All eubacterial RNA-polymerase enzymes described have a five-subunit structure, consisting of a four-subunit core enzyme made up of a single β- and a single β'-subunit (each with an M_r of close to 150000)and two α-subunits (M_r close to 40000). The holoenzyme includes a σ-subunit (M_r close to 90000) which determines the specificity of transcription initiation. Bacterial species which show differentiation during growth phases, such as B.subtilis possess different σ-factors at different stages of growth and sporulation.

The total M_r of the holoenzyme is usually approximately 500000.

The physico-chemical properties of this enzyme have been utilized in its purification. Burgess & Jendrisak (1969) used the differential elution of the enzyme from polyethylene-imine (Polymin P) precipitated cellular DNA, RNA, and protein with increasing salt concentrations to purify the E.coli enzyme. The binding of the RNA-polymerase to polycations, such as heparin, was exploited in the purification method of Davison et al. (1979). Further purification procedures have exploited the large size of the molecule in density gradient centrifugation or gel filtration chromatography.

The anaerobic Archaebacteria have been shown to have RNA-polymerase enzymes which differ from that described in the Eubacteriales, but which show marked similarities to the equivalent enzymes of eukaryotic cells (Zillig et al., 1982).

The bacteriocin described in Chapter 2 was reported to inhibit the synthesis of RNA in sensitive cells without inhibiting DNA synthesis (Mossie et al., 1979). Resistance of derivatives of B.fragilis BF-2 to this inhibition of RNA synthesis was found by Mossie et al. (1980) to be correlated with resistance to rifampicin, which is known to inhibit the action of RNA-polymerase (Schultz & Zillig, 1981). The bacteriocin was also found to inhibit RNA synthesis by RNA-polymerase enzymes from B.fragilis and E.coli in an in-vitro assay (Mossie et al., 1981).

As the project began with an analysis of the bacteriocin produced by B.fragilis BF-1, the RNA-polymerase enzyme of B.fragilis BF-2 was purified and its structure

MATERIALS AND METHODS

B.fragilis BF-2 cells grown in Bacteroides broth to late log phase were collected by centrifugation from a 5000mℓ culture, washed in 0.15M NaCl, and resuspended in 100ml of a solution containing, 0.05M Tris-HCl (pH 7.9), 0.2mM EDTA, 0.23M NaCl, 10% (v/v) glycerol, 50mg lysozyme (Sigma), 16uℓ 2-mercaptoethanol, 10mg phenylmethylsulphonyl fluoride and 1.5mg dithiotreitol, for 30min on ice. The cells were lysed by addition of 50ml TEGD (0.05M tris-HCl, pH 7.9, 0.5mM EDTA, 1mM dithiothreitol, 25% (v/v) glycerol) containing 1% (v/v) Nonidet P40 (Shell Chemicals), and gentle mixing for 20min.

The cellular macromolecules were precipitated by addition of 50ml TEGD containing 5% (v/v) polymin P (Rohm & Haas) while stirring at 4-10°C.

The precipitate was collected, resuspended by grinding with a Silverson high-shear grinder (Janisch SA) in 100ml TEGD containing 0.4M NH_4Cl. The pellet was collected and resuspended in TEGD containing 0.8M NH_4Cl. The supernatant was collected and the enzyme activity precipitated with two volumes of saturated ammonium sulphate. The precipitate was redissolved and dialysed against TEGD containing 10mM $MgCl_2$ and 150mM KCl.

This crude extract was loaded onto a 20ml Heparin-Ultrogel (LKB-IBF Chemicals) column which was used as described by Davison et al. (1979) and the activity was eluted with a buffer containing a linear (0.15 - 1.05M) KCl gradient.

Fractions from the Heparin-Ultrogel column were collected and assayed for RNA-polymerase activity by the method of Robb *et al*. (1977). Active fractions were pooled, concentrated by precipitation with two volumes of saturated ammonium sulphate, redissolved and dialysed against TEGD buffer containing 0.5M KCl.

This was then loaded onto a 10 X 400mm Sepharose 4B (Pharmacia Corp.) column in TEGD containing 0.5M KCl and eluted with the same buffer at 10mℓ/h. Fractions of 2mℓ were collected and assayed for RNA-polymerase activity. Active fractions were analysed by SDS-PAGE on 5 - 15% polyacrylamide gradient gels. (O'Farrell, 1975).

RNA-polymerase was extracted from *E.coli* C600 cells by the method of Burgess & Jendrisak (1969).

RESULTS AND DISCUSSION

A yield of 60 to 75 g wet weight of bacteria was obtained from the 5000mℓ culture, and this was extracted as described. The RNA-polymerase activity was eluted from the Polymin P precipitate by 0.8M KCl but not by 0.4M KCl.

A single peak of activity was eluted by the KCl gradient from the Heparin-Ultrogel (Fig. 1A). Two peaks of activity in the RNA-polymerase assay were obtained from the Sepharose 4B column (Fig. 1B) but only one of these showed the typical eubacterial RNA-polymerase subunits following SDS-PAGE (Fig. 2).

The M_r of the subunits of the B.fragilis RNA-polymerase core enzyme were estimated from the SDS-PAGE with reference to the known sizes of the E.coli enzyme subunits as 155000, 150000 and 40000 respectively. These are very nearly identical to the M_r of the subunits of the E.coli enzyme, as can be seen in Fig. 2.

The σ-subunit of the B.fragilis enzyme was readily dissociated from the core enzyme under the described purification conditions and it was not possible to obtain indisputable evidence of the molecular size of this polypeptide. Most gels showed a weak band with a M_r of approximately 100000 in the most purified fractions, but this was lost from the samples, even in the presence of the SDS and mercaptoethanol of the gel loading buffer.

Reduction of the salt content of buffers used in this extraction and purification of the enzyme below 0.15M resulted in the precipitation and degradation of the enzyme.

This precipitation may be due to the relative absence of the σ-subunit, the absence of which in purified E.coli RNA-polymerase results in the formation of large aggregates of core enzyme at low ionic strengths (Kumar 1981).

The RNA-polymerase from E.coli was readily extracted using the method of Burgess & Jendrisak (1969) and was partially purified using these methods (Fig. 2).

The RNA-polymerase from B.fragilis is essentially similar to that extracted from E.coli, but is more labile under these extraction conditions.

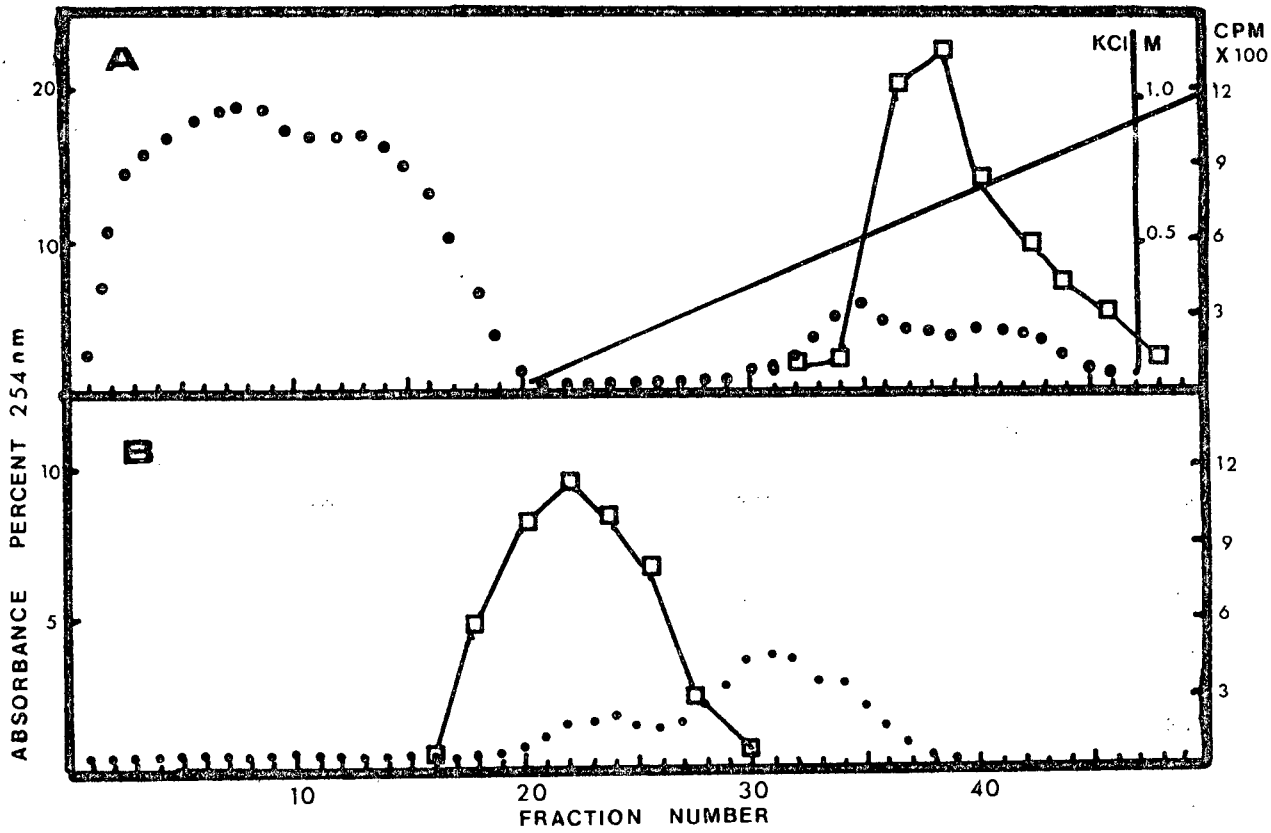


FIG. 1: Chromatographic purification of the RNA-polymerase enzyme from B.fragilis. A. Elution profile from Heparin-Ultrogel; OD_{254} (•••), RNA-polymerase activity (□) and KCl concentration (—). B. Elution profile from Sepharose 4B; OD_{254} (••••) and RNA-polymerase activity (□).

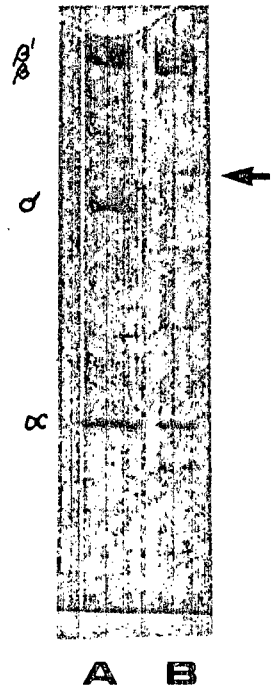


FIG. 2: SDS-polyacrylamide gradient (5-15%) gel electrophoresis of purified samples of RNA-polymerase from (A) E.coli, and (B) B.fragilis. The E.coli subunits are indicated. The putative B.fragilis σ-subunit is marked with an arrow. M_r markers X 10^3. The gel is stained with coomassie brilliant blue R250.

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