THE RELATIONSHIP BETWEEN PLASMID PRESENCE, ANTIBIOTIC RESISTANCE AND SURFACE STRUCTURES IN BACTEROIDES

by

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To the Glory of
GOD
without whom all
this would not have
been possible

Thou spreadest a table before me in the presence of my enemies;
Thou annointest my head with oil, my cup runneth over.

Psalm 23: 5

DECLARATION

The investigations and procedures described in this thesis were performed by the author unless indicated otherwise in the Acknowledgements.

Michelle AE Hamilton

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ABSTRACT

One hundred and fifty-one Bacteroides isolates were collected from a variety of clinical samples (wound, swabs, vaginal swabs and blood cultures) and from the cervix of women attending a colposcopy clinic.

A method for the isolation of plasmid DNA from Bacteroides species was developed and provided a simple and reproducible technique for the analysis of the plasmid content of the isolates. The strains were divided into three groups, the B. fragilis group, the melaninogenicus/oralis group and the asaccharolytic group. The incidence of plasmids in each group was determined.

Forty per cent of the strains were found to have plasmids but there was great variation in the plasmid distribution within each group. Sixy-one percent of the B. fragilis strains had plasmids as opposed to 23% of the asaccharolytic Bacteroides, and 10% of the melaninogenicus/oralis strains. Plasmids ranging in size from 1.1 to 108 MegaDaltons (MDa) were detected but most of the plasmids were less than 10 MDa. Seventy-nine percent of the plasmid-containing strains had more than one plasmid.

The susceptibility of the isolates to chloramphenicol, clindamycin, metronidazole, erythromycin, imipenem, moxalactam, cefoxitin, cefotetan, cefotaxime, cefuroxime, penicillin and nalidixic acid was investigated. Chloramphenicol was the most active non-beta-lactam antibiotic. Six percent of the strains were resistant to clindamycin and metronidazole resistance, though rare,

was observed in 2% of the strains. Among the beta-lactam antibiotics, imipenem, though not in use in the United Kingdom was found to have excellent activity against the Bacteroides. Moxalactam and cefoxitin were also very active with less than 1% of the strains displaying resistance. Various levels of resistance, ranging from 4 to 50% were found amongst the three Bacteroides groups. There was evidence of species variation in antimicrobial susceptibility.

Attempts were made to correlate plasmid presence with observed antibiotic resistance and capsule variation within the strains. Transferable macrolide-lincosamide-streptogramin (MLS) resistance determinants were not identified and observed MLS resistance could not be cured. However, a possible correlation was found between plasmid content and resistance to cefoxitin but plasmid content was not found to alter the resistance pattern of strains to nalidixic acid. Capsule variation was found to depend on altered gene expression and not on the presence or absence of plasmids.

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LIST OF ABBREVIATIONS

h	hour
min	minute
w/v	weight/volume
v/v	volume/volume
EYA	egg yolk agar
BA	blood agar
LBA	lysed blood agar
CBA	combined blood agar
CMB	Robertson's cooked meat broth
PPY	Proteose peptone yeast extract
PPYG	Proteose peptone yeast extract with 10% glucose
DNA	Deoxyribonucleic acid
MDa	Mega-dalton
Mr	Molecular mass
CTX	cefotaxime
CTN	cefotetan
CFX	cefoxitin
CXM	cefuroxime
Cln	Clindamycin
Cm	Chloramphenicol
Erm	Erythromycin
IMP	Imipenem
Mz	Metronidazole

MOX	Moxalactam
NAL	Nalidixic acid
PEN	Penicillin
CAT	Chloramphenicol acetyltransferace
K_m	
MLS	Macrolide, lincosamide streptogramin
MLR^r	Macrolide lincosamide streptogramin resistant
R	Substituents
PBPs	Penicillin binding proteins
MIC	Minimum inhibitory concentration
pI	Isoelectric point
MIC 90	Minimum inhibitory concentration of 90% of the strains tested
MIC 50	Minimum inhibitory concentration of 50% of the strains tested
Kb	Kilobase
DRS	Direct repeat sequences
G + C	Guanidine and cytosine
Cc^r	Clindamycin resistant
Tc^r	Tetracycline resistant
Erm^r	Erythromycin resistant
NCTC	National collection of type cultures
DST agar	Diagnostic sensitivity test agar

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

A. THE IMPORTANCE OF BACTEROIDES IN HUMAN ANAEROBIC INFECTIONS

1. Anatomical Distribution of Bacteroides Species

Bacteroides are obligately anaerobic, non spore-forming, non-motile, Gram-negative rods. Studies of various anatomical sites together with improved anaerobic techniques have given more knowledge of the normal anaerobic flora and of their importance in human infections. A complex anaerobic flora is to be found in the gastrointestinal tract, the female genital tract, the mouth and the upper respiratory tract.

1.1 Bacteroides in the Gastrointestinal Tract

Ninety to ninety-five per cent of the bacteria in the large intestine are obligate anaerobes, while the remainder are coliform or other facultative bacteria. Species belonging to the Bacteroides fragilis group form the predominant flora, being present in the range of 1×10^{10} to 5×10^{10} organisms per gram of faeces (Moore et al., 1969; Stiffler et al., 1974; Guiney and Davis, 1975; Tinnell and Macrina, 1976; Mancini and Behme, 1977; Duerden, 1980b; Rotimi et al., 1981). Some members of this group can occur as opportunist pathogens.

Bacteroides vulgatus and Bacteroides distasonis are the most prevalent members of the Bacteroides fragilis group in the normal flora. However, Bacteroides fragilis, a minor component of the

group, is the major isolate in clinical conditions. It is responsible for more than 60% of all anaerobic infections in humans (Gorbach and Bartlett, 1974a and b; Nastro and Finegold, 1972; Mancini and Behme, 1977; Welch et al., 1979; Wallace et al., 1981; Smith et al., 1982). Infections with this organism occur when predisposing events such as intraabdominal pathology and gastrointestinal or gastro-urinary surgery lead to a breakdown of the normal anatomic barriers (Stiffler et al., 1974; Wallace et al., 1981).

1.2 Bacteroides in the Female Genital Tract

Reports differ as to the prevalence of the Bacteroidaceae in the genital tract. Bacteroides of the melaninogenicus/oralis group have been recently recognised as frequent members of the normal microflora of the lower genital tract (Taylor et al., 1982; Tabaqchali et al., 1983). Of these Bacteroides bivius and disiens are the most common isolates (Duerden, 1980; Evaldson et al., 1982; Larsen and Galask, 1982; Hill and Ayers, 1985).

Although Bacteroides fragilis has been isolated from women with a variety of genital tract infections, the Bacteroides fragilis group is not part of the normal commensal flora of the vagina (Bartlett et al., 1977; Duerden, 1980; Larsen and Galask, 1982; Taylor et al., 1982; Spiegel et al., 1980).

Bacteroides fragilis is present at this site in less than ten per cent of healthy women. It has been suggested that its apparent disproportionate prevalence in genital tract infections is due either

to its presence in low numbers in the vagina or to intermittent contamination of the vagina from the anus. Subsequent multiplication and invasion could then occur when facilitated by local conditions (Bartlett et al., 1977; Levison et al., 1977).

1.3 Bacteroides in the Mouth and Upper Respiratory Tract

Bacteroides species other than those of the Bacteroides fragilis group are usually found in the mouth and upper respiratory tract (Gorbach and Bartlett, 1974b; Duerden, 1980b; Evaldson et al., 1982). They are a minor component of the flora of the healthy gingiva but are isolated in increased numbers during gingivitis (Newman, 1979; Evaldson et al., 1982). Bacteroides gingivalis forms 25-50% of the cultivable flora in advanced periodontitis and is also present in acute periodontitis (Newman, 1979; Evaldson et al., 1982). The predominant Bacteroides in juvenile periodontitis are members of the melaninogenicus/oralis group and there is an absence of Bacteroides gingivalis (Evaldson et al., 1982).

B. PLASMIDS IN BACTEROIDES

1.1 Properties of Plasmid DNA and Incidence in Bacteria

Plasmids are stable extrachromosomal genetic elements which usually exist in the cell as closed, circular deoxyribonucleic acid (DNA) molecules. They replicate autonomously and are known to confer a variety of phenotypic properties of both clinical and ecological significance. Such traits include resistance to antibiotics and

heavy metals (Helinsky, 1973; Summers and Lewis, 1973), toxin production including enterotoxins (Gyles et al., 1974), exfoliative toxins (Warren et al., 1975) and haemolysins (Goebel et al., 1974; Jacob et al., 1975), surface antigens (Bak et al., 1972), bacteriocins and hydrocarbon catabolism (Macrina and Balbinder, 1973; Jacob et al., 1975), production of sex factors for conjugation (Pemberton and Clark, 1973) and the use of organic compounds such as camphor and napthalene (Dunn and Gunsalus, 1973).

Because of the importance of several of these characteristics to human disease and microbial ecology, plasmids have been sought and found in a wide variety of bacteria including Pseudomonas (Keil and Williams, 1985; Sharabadi et al., 1975; Takahashi and Nagano, 1984), Bacillus (Lovett,1973), Staphylococcus (Takahashi and Nagano, 1984) and most genera of the Enterobacteriaceae (Goebel et al., 1974; Takahashi and Nagano, 1984). The ecological niche of the Bacteroides, their potentially important role in human intestinal pathology and their importance as opportunistic pathogens, have made members of this genus the subject of much current investigation.

1.2 The Incidence of Plasmids in Bacteroides

Plasmids have been demonstrated in most representatives of the Bacteroides fragilis group in the fragilis species as well as other species. Young and Mayer (1979) in their analysis of 62 strains of Bacteroides isolates found that only 46% possessed plasmids. This figure was similar to that of Riley and Mee (1984) who examined 50 isolates, but is somewhat higher than the 26% found by Wallace et al. (1981) who studied 32 random Bacteroides isolates. Stiffler et al.

(1974) found plasmids in three of four clinical isolates of Bacteroides. Most strains of Bacteroides examined carried a multiple plasmid population. Of 40 plasmid-containing strains examined by 11 groups of workers 26 (65%) contained more than one plasmid band.

1.3 The Size Range of Plasmids in Bacteroides

The overall size range of plasmids isolated from Bacteroides species was between 2.0 and 58 megadaltons (MDa), with the most commonly found plasmids having molecular masses of less than 10MDa. Wallace et al. (1981) found three distinct sets of plasmids of sizes 2.0, 3.0 and 5.0MDa, while Young and Mayer (1979) found that 26 of their 29 plasmid-containing strains had plasmids with molecular weights of less than 10MDa. In their study of 50 Bacteroides strains, Riley and Mee (1984) found plasmids of 9.8MDa and less in 18 of 20 plasmid-containing strains. Plasmids with molecular masses of greater than 10Md have been found in Bacteroides fragilis by Stiffler et al. (1974), Guiney and Davis (1975), Welch et al. (1981), Guiney et al. (1984), and Smith and Macrina (1984). Riley and Mee (1981) found a plasmid greater than 10MDa in Bacteroides distasonis, while Tinnell and Macrina (1976) and Rotimi et al. (1981) found plasmids greater than 10MDa in Bacteroides thetaiotaomicron. Martinez-Duarez et al. (1985) isolated a 23.7MDa plasmid from a strain of Bacteroides uniformis.

This size range of plasmids in Bacteroides is rather small by comparison with aerobes such as Pseudomonas and facultative

anaerobes such as Escherichia coli from which plasmids over 80MDa have been isolated (Takahashi and Nagano, 1984) (Table 1.1).

C. ANTIBIOTIC THERAPY OF BACTEROIDES INFECTIONS

The term antibiotic/antimicrobial agent is used throughout this thesis to include synthetic chemotherapeutic agents as well as true antibiotic substances produced by micro-organisms.

1. Therapeutic Problems of Bacteroides Infections

Infections with Bacteroides pose many therapeutic problems because of their resistance to commonly used antimicrobial agents. Some classes of antibiotics such as the aminoglycosides are known to be inactive against these species (Watt, 1979; Bryan and Kwan, 1981; Wexler and Finegold, 1987). Other classes such as the tetracyclines are becoming increasingly ineffective due to the emergence and spread of resistant strains. Use is recommended only where laboratory tests demonstrate susceptibility of the isolates (Nastro and Finegold, 1972; Chow et al., 1975; Sutter and Finegold, 1976; Brown and Waatti, 1980; Snydman et al., 1980; Cuchural et al., 1981; Phillips et al., 1981).

Clindamycin (Cc) and chloramphenicol (Cm) were traditionally used for the treatment of bacteroides infections. The unique antimicrobial activity of metronidazole (Mz) has only recently been recognised. An increased awareness of the importance of anaerobic bacteria in infections has emphasised the need for new antimicrobial agents. In recent years considerable effort has gone into the

TABLE 1.1

Molecular masses (Mr) of plasmids detected in various clinical isolates*

Strain tested	Mr of plasmids detected (MDa)
Escherichia coli CU06711	83, 44
E. coli CU09232	72, 42, 2.7
Citrobacter freundii CU17160	100, 3.9, 2.0
C. freundii CU13222	120, 48
Enterobacter cloacae CU06407	100, 83, 48, 2.6, 2.0
E. aerogenes CU13116	120, 100, 21, 2.7
Serratia marcescens CU16741	100, 63, 42, 20, 2.3
Klebsiella pneumoniae CU03092	100, 68
K. pneumoniae CU06351	60, 32, 2.5
Proteus vulgaris CU08806	100, 83, 2.6, 2.0
Morganella morganii CU23232	36, 28
Providencia rettgeri CU21322	115, 90
Pseudomonas aeruginosa CU13325	85, 45, 3.5, 3.3
P. aeruginosa CU20304	117, 54, 16
Haemophilus influenzae FM0634	43
Staphylococcus aureus FM0333	38, 11, 2.1

* Data of Takahashi and Nagano (1984)

development of beta-lactam antibiotics which show improved activity by virtue of resistance to beta-lactamases produced by many species of aerobic and anaerobic bacteria.

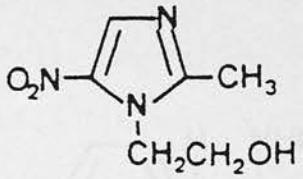
2. Non-Beta Lactam Antibiotics with Activity Against Bacteroides

2.1 Metronidazole

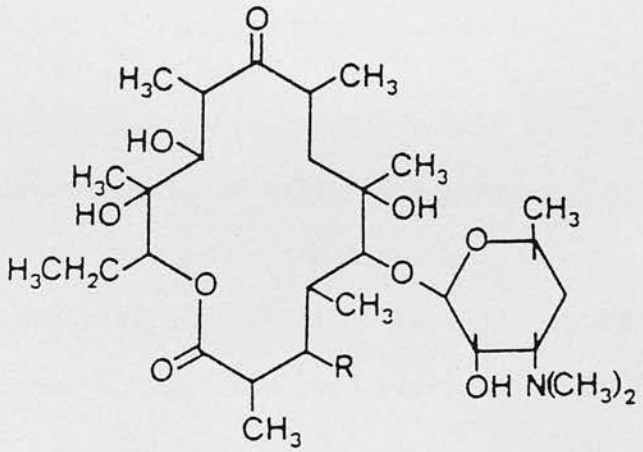
Metronidazole is a synthetic derivative of the nitroamidazoles, a group of heterocyclic compounds based on a 5-membered nucleus (Fig 1.1a). It was originally introduced for the treatment of trichomoniasis but has gained widespread use for both the prophylaxis and treatment of anaerobic infections (Baines, 1978; Eykyn and Phillips, 1978).

Metronidazole is selectively active against obligate anaerobes and clinical experience has shown it to be highly effective against Bacteroides (Nastro and Finegold, 1972; Sutter and Finegold, 1976; Cuchural et al., 1981; Ohm-Smith et al., 1982). Nitro compounds have, however, been reported to be weakly mutagenic and carcinogenic (Finegold, 1977; Roe, 1977) and fears have been expressed about the saftey of metronidazole (Anon, 1975). Investigations have shown that while there is a theoretical risk associated with its usage metronidazole is one the safest nitro compounds for the treatment of anaerobic infections (Roe, 1977; Reynolds, 1981).

(a) Metronidazole



(b) Erythromycin



(c) Clindamycin

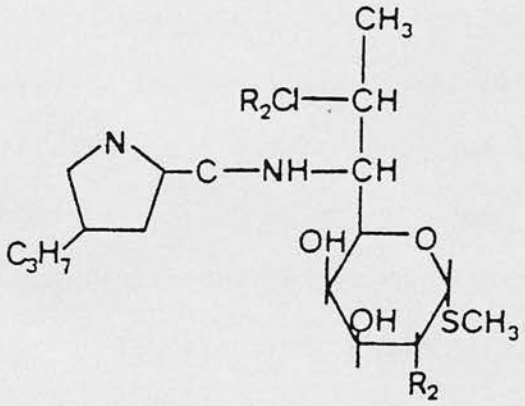
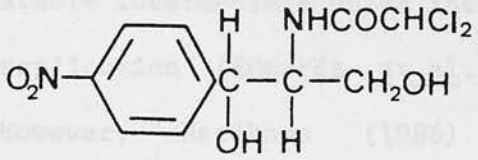


Figure 1.1

Structures of some non-beta lactam antibiotics with activity against Bacteroides

Figure 1.1 (continued)



(d) Chloramphenicol

2.1.1 Mode of Action of Metronidazole

The mode of action of this bacteriocidal drug is not clear. It was thought to involve reduction by nitroreductases to an uncharacterised, unstable intermediate which interacted with DNA and prevented further replication (Edwards *et al.*, 1973; Chrystal *et al.*, 1980). However, Narikawa (1986) showed that the nitroreductase activity of bacteria may not directly influence metronidazole susceptibility but may affect drug uptake.

Evidence that pyruvate ferredoxin oxidoreductase activity is the major factor in metronidazole susceptibility was presented by O'Brien and Morris (1972). This was later confirmed by several workers including Britz (1981) and Narikawa (1986). In 1983 the subject was reviewed by Mueller who put forward the hypothesis shown in Fig 1.2.

2.1.2 Resistance to Metronidazole

In the UK resistance to metronidazole is rare and few reports have been substantiated (Ingham *et al.*, 1978; Rotimi *et al.*, 1979; Eme *et al.*, 1983). However, in France 12% of the clinical isolates investigated by Acar *et al.* (1981) were resistant to 8 mg/l of metronidazole. In South Africa 21% of *Bacteroides fragilis* were reported by Appelbaum and Chatterton (1978) to be resistant to 16 mg/l metronidazole; and in the USA resistance to 100 mg/l metronidazole in 17% of *Bacteroides* species was reported by Chow *et al.* (1975).

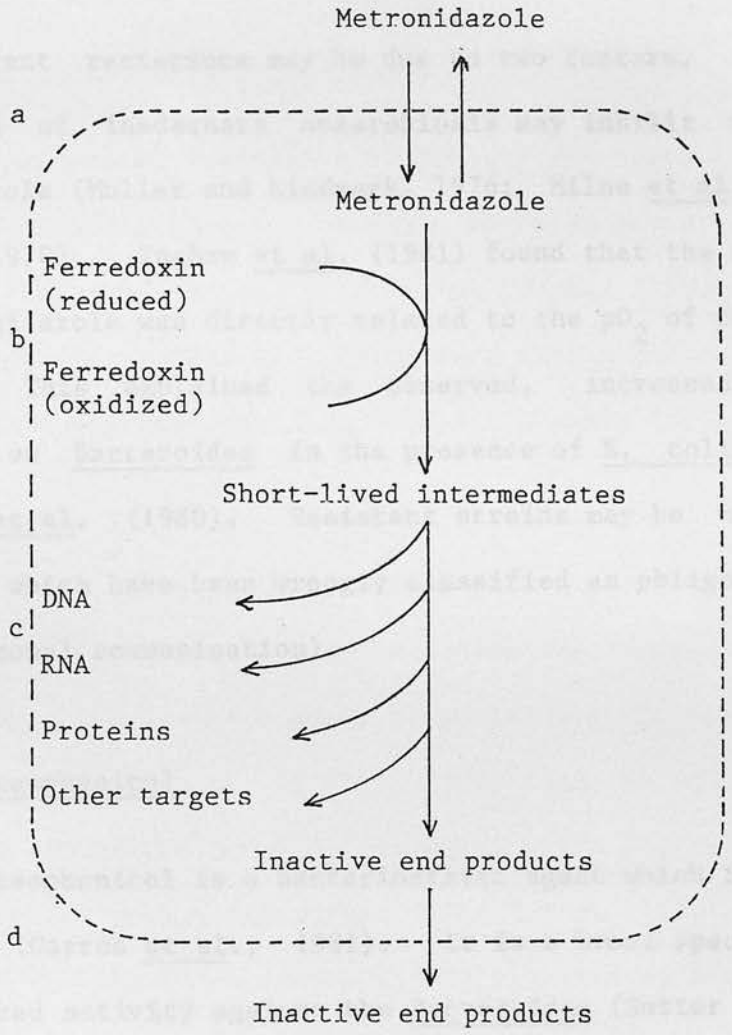


Figure 1.2

Scheme of action of metronidazole on an anaerobic microorganism: a, passage through the cell membrane; b, reductive activation; c, interaction with intracellular targets; d, release of inactive end products (Mueller, 1983).

Apparent resistance may be due to two factors. Testing under conditions of inadequate anaerobiosis may inhibit the action of metronidazole (Muller and Lindmark, 1976; Milne et al., 1978; Tally et al., 1978). Ingham et al. (1981) found that the rate of killing by metronidazole was directly related to the pO_2 of the surrounding medium. This explained the observed, increased bacteriocidal activity on Bacteroides in the presence of E. coli described by Chrystal et al. (1980). Resistant strains may be microaerophillic organisms which have been wrongly classified as obligate anaerobes (B Watt, personal communication).

2.2 Chloramphenicol

Chloramphenicol is a bacteriostatic agent which inhibits protein synthesis (Garrod et al., 1981). It is a broad spectrum antibiotic with marked activity against the Bacteroides (Sutter et al., 1973; Sutter and Finegold, 1976; Kaye et al., 1980; Snydman et al., 1980; Cuchural et al., 1981; Phillips et al., 1981; Ohm-Smith et al., 1982). With the large scale emergence of tetracycline-resistant strains, chloramphenicol replaced tetracycline as the initial agent for therapy of bacteroides infections (Bodner et al., 1970; Tally et al., 1979). However, it may produce dangerous bone marrow depression or aplasia (Garrod et al., 1981). As a result, at present one of its limited uses is in the treatment of serious infections caused by Bacteroides fragilis.

2.2.1 Resistance to Chloramphenicol

Chloramphenicol resistance in Bacteroides species is a very rare occurrence (Phillips et al., 1981; Martinez-Suarez et al., 1985). Of 1,266 isolates examined by Brown and Waatti (1980) only 25 (2%) were found to be resistant. Resistance may be due to impermeability or to enzymic degradation (Nagai and Mitsuhashi, 1972; Shaw, 1975).

The molecule can be attacked at a number of different points by bacterial enzymes and the degradation products have no antibacterial activity. Chloramphenicol nitroreductase inactivates chloramphenicol by reducing the p-nitro group at position 6 of the benzene ring (Britz and Wilkinson, 1978b; Tally and Malamy, 1984). More frequently inactivation of chloramphenicol is due to acetylation by chloramphenicol acetyltransferase (CAT) (Britz and Wilkinson, 1978b; Britz, 1981; Martinez-Suarez, 1985). This enzyme utilises acetyl-coenzyme A as an acyl donor and produces a 3-monoacetoxy derivative of chloramphenicol as the major product.

CAT enzymes have been detected in Bacteroides fragilis and Bacteroides uniformis (Britz and Wilkinson, 1978b; Britz, 1981; Martinez-Suarez, 1985). They were similar to CAT type II from enterobacteria but were nevertheless considered to be distinct. Properties of the CAT enzyme from Bacteroides fragilis are outlined in Table 1.2. Both the K_m (affinity for chloramphenicol) and specific activity were lower than the CAT from Bacteroides uniformis.

TABLE 1.2

Properties of chloramphenicol acetyltransferase from B. fragilis*

(1)	pH optimum for assay of 7.8
(2)	Molecular mass of 89,000 daltons
(3)	Acidic protein (binds to DEAE gels)
(4)	Major product is Cm-3-acetate, some Cm-1 acetate
(5)	Stable to heating at 75°C for 10 min
(6)	Constitutively synthesized
(7)	Specific activity (post heating 70°C, 10 min): 0.03umol/min/mg protein
(8)	K_m for Cm about 5uM
(9)	Sensitive to inhibition by DTNB and p-chloromercuribenzoate
(10)	Unstable during storage

*Data of Britz and Wilkinson (1978b)

2.3 The Macrolide-Lincosamide Group

The macrolides are a large group of antibiotics with similar properties. They consist of a macrocyclic lactone ring to which sugars are attached. Erythromycin is the most widely used. The lincosamides are derivatives of lincomycin, an antibiotic isolated from a strain of Streptomyces lincolnensis (Lewis et al., 1962). The most widely used and most active of these drugs is clindamycin.

2.3.1 Erythromycin

The structure of erythromycin is shown in Fig 1.1b. This drug has a narrow spectrum of activity being mainly active against Gram-positive species. It has, however, some activity against the Bacteroides and was in the past recommended with lincomycin as the drug of choice for severe infections due to Bacteroides fragilis (Finegold et al., 1966; Ingham et al., 1968). Both were, however, replaced by clindamycin which was shown to be more active in vitro (Ingham et al., 1970; Finegold et al., 1972; Nastro and Finegold, 1972).

Erythromycin is primarily bacteriostatic but may be bacteriocidal in higher concentrations (Garrod et al., 1981). Its mode of action is similar to that of clindamycin. Side effects are rare but the usefulness of this antibiotic has been reduced by the emergence of bacterial resistance (Sutter et al., 1973).

2.3.2 Clindamycin

Clindamycin was produced by 7-chloro substitution of the 7 (R) hydroxyl group of lincomycin and is available as a hydrochloride salt, a phosphate or an ester. Clindamycin is a primarily bacteriostatic agent which inhibits protein synthesis (Garrod et al., 1981). It prevents the synthesis of bacterial peptides by acting specifically on the 50S sub-unit of the bacterial ribosome, possibly affecting the process of peptide chain initiation (Cundliffe, 1969; Reuesser, 1975).

Clindamycin shows excellent activity against most anaerobic pathogens (Nastro and Finegold, 1972; Sutter et al., 1973; Sutter and Finegold, 1976; Kaye et al., 1980; Snydman et al., 1980; Ohm-Smith et al., 1982; Van der Auwera et al., 1987). It has been recommended as the drug of choice for treatment of anaerobic infections because of its therapeutic efficacy, ease of administration and lack of resistance (Tally et al., 1979; Reig et al., 1984). The value of clindamycin has, however, been reduced by the development of bacterial resistance and the occurrence of pseudomembranous colitis in some patients treated with the drug (Gorbach and Bartlett, 1974; Finegold, 1977; Finegold 1979).

2.3.3 Resistance to Clindamycin and Erythromycin

Resistance to clindamycin can be low level (5-20 mg/l) or high level (>160 mg/l) (Rahman, 1978; Salakai et al., 1976; Yee et al., 1982; Guiney et al., 1983), and may be the result of two different

mechanisms of resistance in Bacteroides species (Guiney et al., 1983).

High level resistance to clindamycin in anaerobic bacteria is usually associated with resistance to erythromycin and the streptogramins and is known collectively as macrolide, lincosamide, streptogramin (MLS) resistance (Privatera et al., 1981; Guiney et al., 1983). This type of resistance is mediated by methylation of the adenine residues of the 23S ribosomal RNA. The resulting reduced affinity between the MLS antibiotic and the 50S ribosomal sub-unit prevents binding and protein synthesis is not affected (Lai and Weisblum, 1971; Rasmussen et al., 1986). Low level resistance is less prevalent among the Bacteroides. Strains exhibiting this type of resistance show variable cross-resistance to lincomycin and erythromycin (Guiney et al., 1983).

The first report of clindamycin resistance in the Bacteroides fragilis group appeared in 1976 (Salakai et al., 1976). Resistance though uncommon a few years ago has been increasing (Salakai et al., 1976; Acar et al., 1981). There is wide variation in the literature as to the prevalence of resistant strains. Phillips et al. (1981) found 2% of Bacteroides strains isolated from clinical material in a London hosptial were resistant to clindamycin. Brown and Waatti (1980) found 5% of 1,266 isolates in Detroit were resistant. This was similar to the 7% resistance observed by Cuchural et al. (1981) in isolates from Boston, but all were much less than the 20% resistance observed by Acar et al. (1981) during investigation of 717 isolates in France.

Bacteroides fragilis is known to acquire resistance to clindamycin and erythromycin during prolonged treatment or after *in vitro* exposure to these drugs (Soriano, 1981; Soriano *et al.*, 1980). Variation may be due to specific epidemiological factors or to local usage of the macrolide-lincosamide antibiotics.

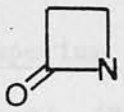
2.4 The Beta-Lactam Antibiotics

The beta-lactam antibiotics include the penicillins, the cephalosporins, the cefamycins and the thienamycins. All contain the fused beta-lactam ring system (Fig 1.3a).

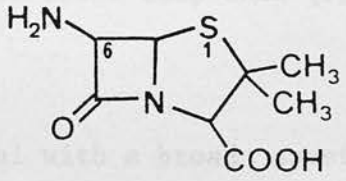
2.4.1 The Penicillins

Penicillin (Pn) is one of the natural products of the mould *Penicillium chrysogenum*. It is active against select Gram-postive and Gram-negative organisms. The nucleus of the penicillin molecule is 6-amino-penicillanic acid (Fig 1.3b). Substitution has produced a variety of antibiotics which have different properties (Fig 1.3c). They are either acid or penicillinase resistant with broad or narrow spectra of antimicrobial activity. All penicillins are bacteriocidal to actively growing cells. They inhibit the transpeptidase step in cell-wall peptidoglycan synthesis (Garrod *et al.*, 1981).

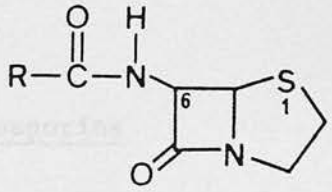
(a) The beta lactam ring



(b) 6-amino-penicillanic acid



(c) Penicillin G



(d) 7-aminocephalosporanic acid

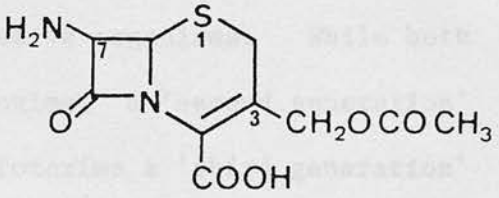


Figure 1.3

Relationship of the penicillins and cephalosporins to the beta-lactam ring. R represents substituents.

2.4.2 The Cephalosporins

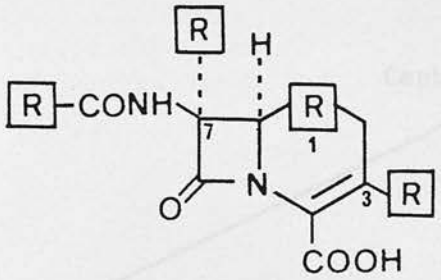
The cephalosporins are synthetic derivatives of cephalosporin C, an antibiotic produced by the mould Cephalosporium acremonium. The basic nucleus is 7-aminocephalosporanic acid (Fig 1.3d) (Garrod et al., 1981). It consists of a dehydrothiazolidine ring fused with a four-membered beta-lactam ring. Modification of the nucleus by substitution at a number of places can affect the antibacterial activity and pharmacological properties of these compounds (Fig 1.4a) (Neu, 1981).

All cephalosporins are bacteriocidal with a broad spectrum of activity. They inhibit cell-wall synthesis in Gram-positive cocci and Gram-negative bacilli by a mechanism similar to that of the penicllins.

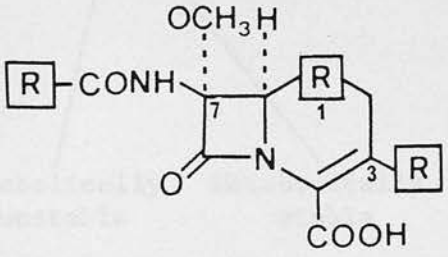
2.4.3 Classification of the Cephalosporins

Cephalosporins for human clinical use are sub-divided into five groups on the basis of the method of administration and beta-lactamase resistance (Fig 1.5)*. Cefuroxime (CXM) and cefotaxime ((HR745) CTX) are both broad spectrum cephalosporins active against a wide range of Gram positive and Gram negative organisms. While both are resistant to beta-lactamase, cefuroxime, a 'second generation' cephalosporin, is less resistant than cefotaxime a 'third generation' cephalosporin (O'Callaghan et al., 1976; King et al., 1980; Jones and Thornsberry, 1982; Neu, 1982). Both have a methoxyaminoside group (Fig 1.6).

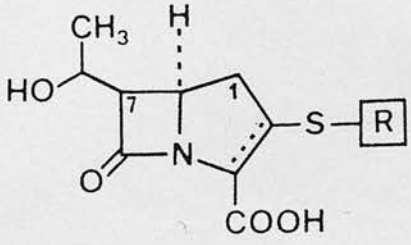
*More recent classifications take account of the anti-pseudomonas activity of these antimicrobial agents.



a Basic nucleus of the cephalosporins (cephem nucleus) with R (representing substituents) at positions in which major modifications are possible. Substitutions at position 3 and/or the acyl side chain alter antibacterial and pharmacological properties. Substitutions at or near position 7 affect stability to beta-lactamase enzymes (Neu, 1982).



b Structure of the cefamycins (cephem nucleus).



c Structure of the thienamycins (carbapenem nucleus).

Figure 1.4

Relationship between the cephalosporins, cefamycins and thienamycins.

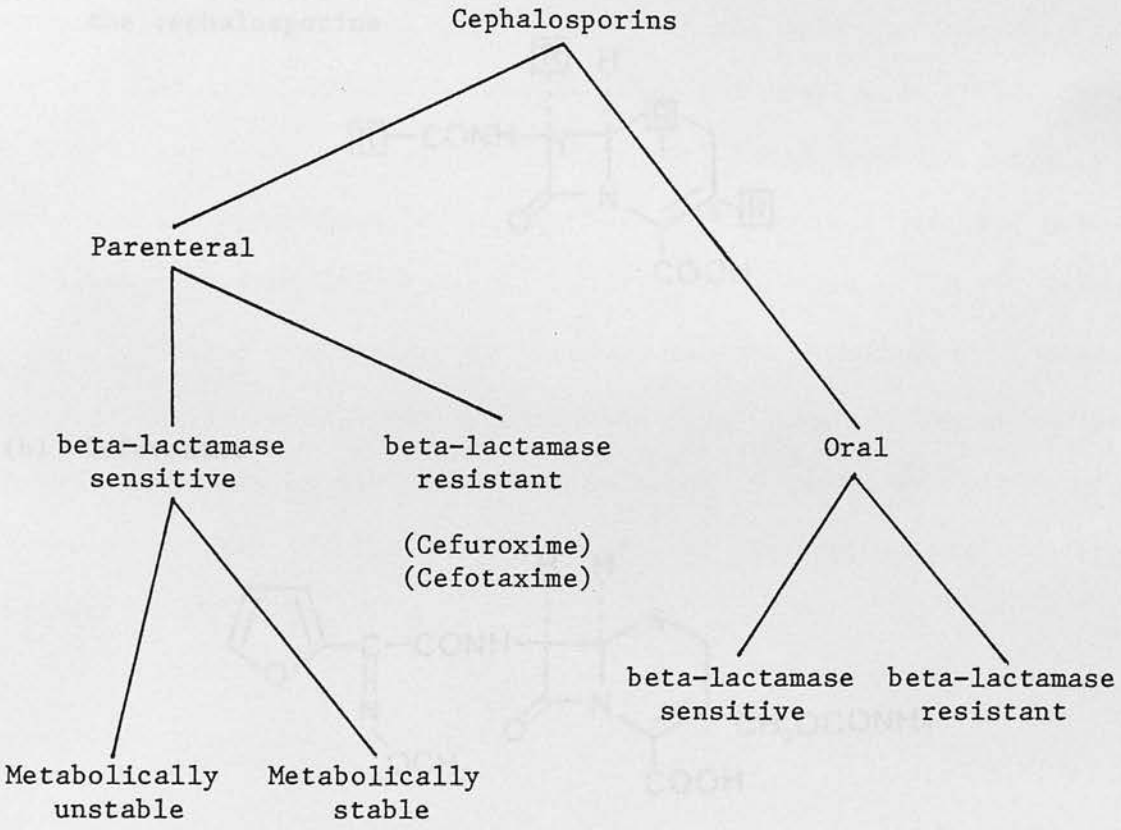
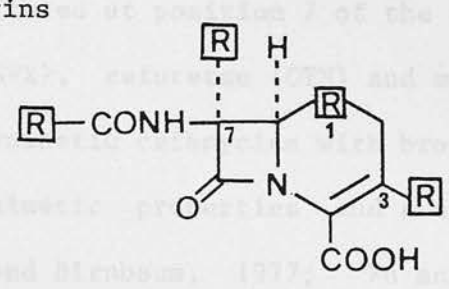


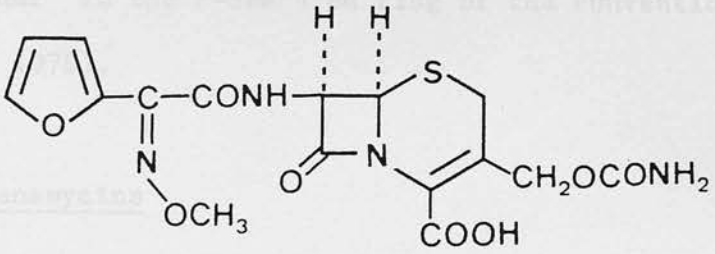
Figure 1.5

Classification of cephalosporins for human clinical use (O'Callaghan, 1979).

(a) Basic structure of the cephalosporins



(b) Cefuroxime



(c) Cefotaxime (HR756)

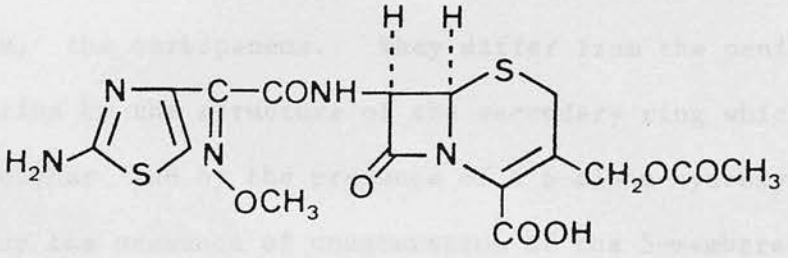


Figure 1.6

Structures of selected cephalosporins

2.4.4 The Cefamycins

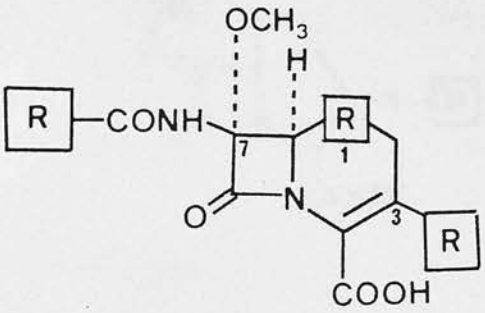
The cefamycins are derivatives of the naturally occurring cefamycin C. They are alpha-methoxy cephalosporins which have a methoxy group substituted at position 7 of the beta-lactam ring (Fig 1.4b). Cefoxitin (CFX), cefotetan (CTN) and moxalactam (MOX) (Fig 1.7) are all semi-synthetic cefamycins with broad spectrum activity, favourable pharmacokinetic properties and are resistant to beta-lactamases (Darland and Birnbaum, 1977; Fu and Neu, 1981; Ayers et al., 1982). Cefotetan is unusual in that tautomeric changes produce small amounts of a tautomer with similar antimicrobial activity (Iwanami et al., 1980). Moxalactam is unique in that oxygen replaces sulphur in the 6-membered ring of the conventional cephem nucleus (Fass, 1979).

2.4.5 The Thienamycins

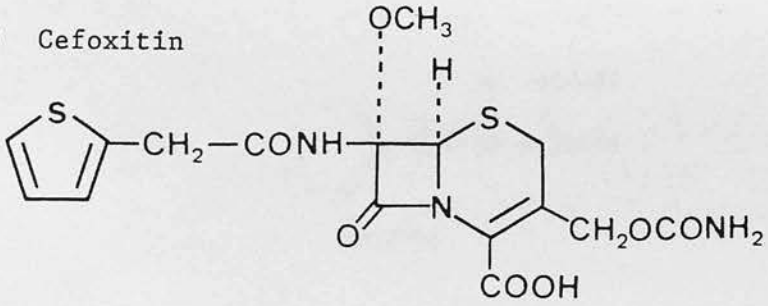
The thienamycins are derivatives of thienamycin, a beta-lactam antibiotic produced by the soil organism Streptomyces cattleya (Kahan et al., 1983). Thienamycin is the first representative of a unique and diverse class of naturally occurring and synthetic beta-lactam antibiotics, the carbapenems. They differ from the penicillins and cephalosporins by the structure of the secondary ring which does not contain sulphur and by the presence of a 6-alpha-hydroxyethyl side chain and by the presence of unsaturation of the 5-membered ring (Fig 1.4c).

N-formimidoyl thienamycin (Imipenem, MK0787) is an amidine derivative of thienamycin (Fig 1.8a). Unlike thienamycin it is

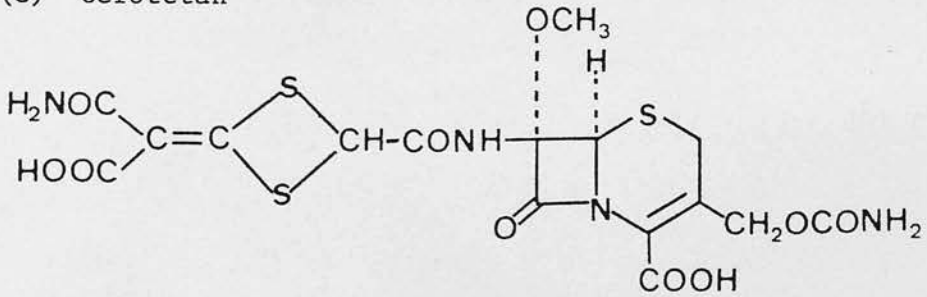
(a) Basic structure of the cefamycins



(b) Cefoxitin



(c) Cefotetan



(d) Moxalactam (Ly127935, 6059-S)

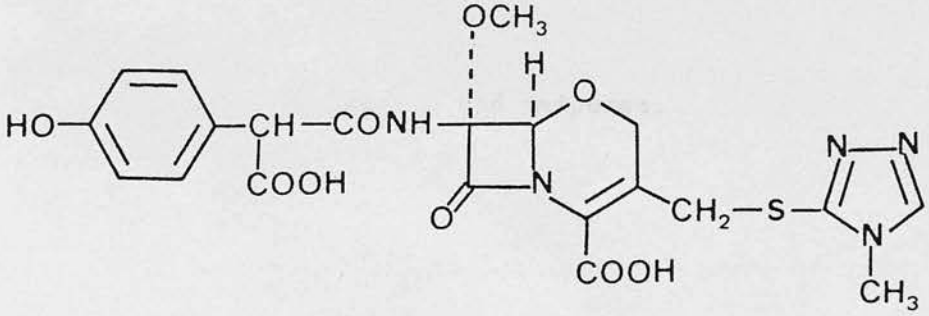
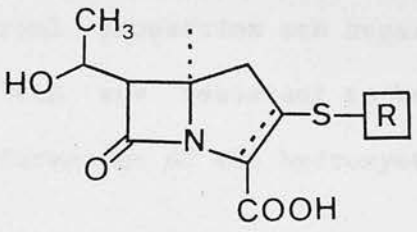
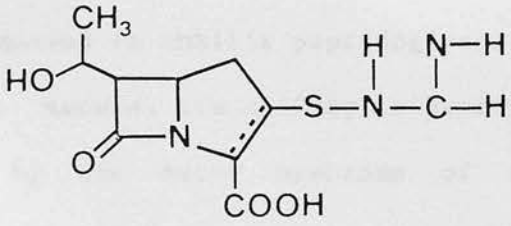


Figure 1.7

Structures of cefamycin antibiotics.



(a) Thienamycin



(b) Imipenem (MK0787)

Figure 1.8

Structures of thienamycin and imipenem.

chemically stable in concentrated solution (Leanza et al., 1979) and stable within the range of pH 6-8 (Borobio et al., 1985). Its antibacterial properties are superior to thienamycin (Kahan et al., 1983). Both are resistant to beta-lactamases due to the unusual trans-conformation of the hydroxyethyl side chain (Cassidy, 1981).

2.5 Resistance to Beta-Lactam Antibiotics in Gram Negative Bacteria

The activity of beta-lactam antibiotics against bacterial species seems to depend on three main properties: first, the ability of the compound to inhibit peptidoglycan synthesis in the cytoplasmic membrane; second, its ability to penetrate the permeability barrier afforded by the outer membrane of the Gram-negative bacterial envelope to reach its target; and third, the compound must be resistant to any beta-lactamases produced by the organisms.

2.5.1 Penicillin Binding Proteins of Bacteroides

The targets of beta-lactam antibiotics are a number of proteins located in the bacterial cytoplasmic membrane. These proteins are penicillin-sensitive enzymes which bind penicillin irreversibly to the bacterial membranes. They are referred to as penicillin binding proteins (PBPs) and their cytoplasmic membrane location indicates involvement in the catalysis of the terminal stages of peptidoglycan synthesis (Spratt, 1977).

PBPs have been detected in several genera of both Gram-negative and Gram-positive bacteria (Spratt, 1975, 1977; Nolan and Hildebrandt, 1979; Zimmerman, 1980; Georgopapadakou et al., 1980,

1983). They have been extensively studied in E. coli for which essential PBPs have been identified and their functions elucidated (Spratt, 1977; Suzuki et al., 1978; Tamura et al., 1980). In recent years there have been investigations into the properties of PBPs in Bacteroides (Botta et al., 1983; Georgopapadakou et al., 1983; Otsuki et al., 1983; Piddock and Wise, 1986).

There is conflicting information in the literature on the number of PBPs in Bacteroides and their molecular masses (Georgeopapadakou et al., 1983; Otsuki et al., 1983). Piddock and Wise (1986) reported the presence of three major PBPs in Bacteroides fragilis, PBP 1, 2 and 3. They observed that PBP 1 could be resolved into a doublet or triplet. An additional PBP, PBP 4, which was found in single isolates of Bacteroides vulgatus, Bacteroides ovatus and Bacteroides thetaiotamicron could be detected occasionally in Bacteroides fragilis under special conditions. Bacteroides vulgatus was also found to have a PBP 5. The molecular masses of the PBPs ranged between 38,000 and 82,000 with the highest molecular mass being that of PBP 1.

Botta et al. (1983) detected only 3 PBPs in two clinical isolates of Bacteroides fragilis with molecular masses ranging between 53,000 and 76,000. De La Rosa et al. (1982) showed that changes in the growth state of E. coli cells resulted in modification of the PBPs. Differences in methodology may therefore account for the discrepancies. Botta et al. (1983) examined cells in the log phase while Piddock and Wise (1986) examined cells in the late log to early stationary phase. It is also possible that more subtle

differences such as growth media, gel composition or oxygen lability of PBPs could explain the contradictory results.

Investigation of the affinities of the PBPs by Piddock and Wise (1986) showed that the higher molecular mass PBPs were involved in binding at concentrations approximating the minimum inhibitory concentrations (MICs). For most compounds the primary target was PBP 2 followed by PBP 1. Differences in bias shown by the remaining compounds were thought to reflect the availability of particular PBPs *in vivo*.

These workers postulated that for the genus *Bacteroides*, the killing targets for most beta-lactam antibiotics were PBPs 1 and 2. A correlation was found between the binding to PBP 1 and the response of spheroplasting and lysis. It was therefore deduced that the components of PBP 1 were enzymes involved in cell elongation. The binding of PBP 2 was correlated with the morphological response of filamentation. Piddock and Wise (1986) further deduced that PBP 2 was involved in septation and PBP 3 in the maintenance of cell shape. In addition, compounds that bound to PBP 1 were found to kill more rapidly than compounds that bound to PBP 2 and PBP 3.

Morphological changes in the cells could not be correlated with the binding to PBPs 4 and 5. These compounds may be similar to the 'dispensable' PBPs of *E. coli* which are non-essential for shape maintenance and cell division (Mutahashi *et al.*, 1977).

2.5.2 Beta-Lactamase Activity of Bacteroides

Beta-lactamases are enzymes which hydrolyse the beta-lactam ring of beta-lactam antibiotics (Fig 1.9). Several authors have established a correlation between the resistance of a number of species of Bacteroides to beta-lactam antibiotics and the production of beta-lactamases.

Beta-lactam activity in Bacteroides was first described by Garrod (1955). Since then several investigators have reported the presence of beta-lactamases in varying percentages of strains of the Bacteroides fragilis group. These percentages range from 50 to 95% and possibly reflect differences in methodology (Weinrich and Del Bene, 1976; Darland and Birnbaum, 1977; Olsson et al., 1977; Gabay et al., 1981; Nord and Olsson-Liljequist, 1981; Eley and Greenwood, 1986). Beta-lactamase activity has also been detected in Bacteroides of the melaninogenicus/oralis group (Pinkus et al., 1968; Hackman and Wilkins, 1976; Salyers et al., 1977).

Characterisation of B-lactamases in Bacteroides Species

The beta-lactamases of Bacteroides are a new class of enzymes. They do not fit into the existing classification scheme worked out for aerobic Gram-negative rods by Richmond and Sykes (1973) (Olsson et al., 1976; Maskell et al., 1984).

Substrate Profile

The Bacteroides fragilis group produces more than one type of beta-lactamase. Most have predominantly cephalosporinase activity

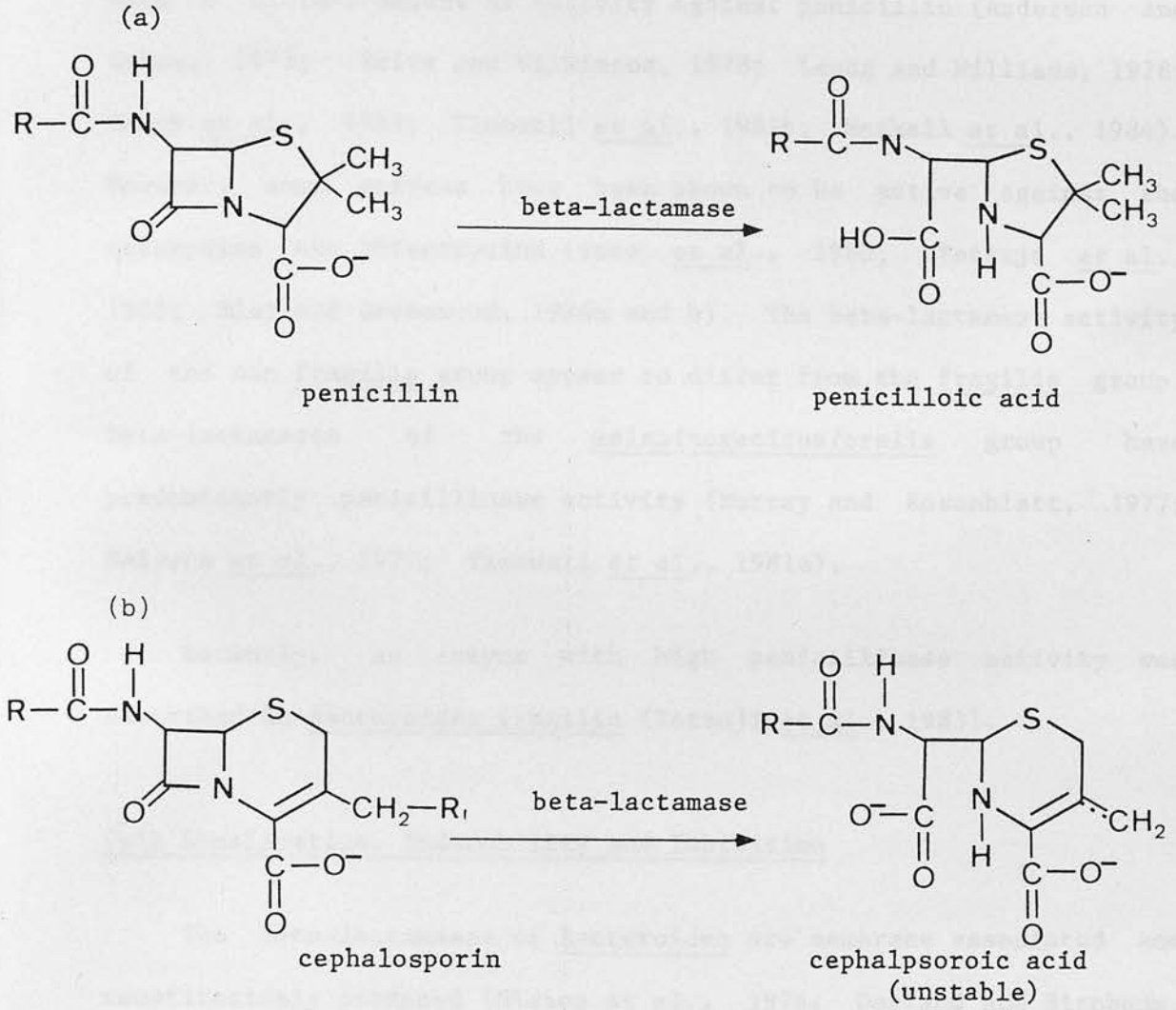


Figure 1.9

Beta-lactam hydrolysis of the beta-lactam bond in (a) the penicillin molecule to form the biologically inactive penicilloic acid, (b) the cephalosporin molecule to form the inactive, unstable cephalosporoic acid which gives rise to substrate dependent products (Richmond and Sykes, 1973). R and R_1 indicate substituents.

with a minimal amount of activity against penicillin (Anderson and Sykes, 1973; Britz and Wilkinson, 1978; Leung and Williams, 1978; Gabay et al., 1981; Timewell et al., 1981b; Maskell et al., 1984). However, some enzymes have been shown to be active against the cefamycins and thienamycins (Sato et al., 1980; Yotsuji et al., 1983; Eley and Greenwood, 1986a and b). The beta-lactamase activity of the non fragilis group appear to differ from the fragilis group. Beta-lactamases of the melaninogenicus/oralis group have predominantly penicillinase activity (Murray and Rosenblatt, 1977; Salyers et al., 1977; Timewell et al., 1981a).

Recently, an enzyme with high penicillinase activity was described in Bacteroides fragilis (Yotsuji et al., 1983).

Cell Localisation, Inducibility and Inhibition

The beta-lactamases of Bacteroides are membrane associated and constitutively produced (Olsson et al., 1976; Darland and Birnbaum, 1977; Britz and Wilkinson, 1978; Sherrill and McCarthy, 1979). Their activity is highly dependent upon culture age. Synthesis occurs during exponential growth, reaching a maximum in mid-to-late log phase. Rapid decline in activity occurs during the stationary phase (Olsson et al., 1976; Darland and Birnbaum, 1977; Britz and Wilkinson, 1978).

The enzymes produced by the Bacteroides fragilis group are usually susceptible to most of the common inhibitors, cloxacillin, p-chloromercuribenzoate, clavulanic acid, sublactam and 6-B-iodopenicillanic acid (Wise et al., 1980; Nord and Olsson-

Liljequist, 1981; Smith et al., 1982; Maskell et al., 1984). However, enzymes resistant to one or more of these inhibitors have been reported (Yotsuji et al., 1983; Eley and Greenwood, 1986a and b). Enzymes produced by the melaninogenicus/oralis group are inhibited by clavulanic acid but not by cloxacillin or p-chloromercuribenzoate (Wust and Wilkins, 1978; Timewell et al., 1981a).

Molecular Weights

Three ranges of molecular masses have been reported for the beta-lactamases of Bacteroides fragilis. Values between 20,000 and 30,000 were reported by Britz and Wilkinson (1978) and Yotsuji et al. (1983); between 30,000 and 40,000 by Olsson et al. (1976), Sato et al. (1980) and Simpson et al. (1982); and greater than 40,000 by Olsson et al. (1976). Timewell et al. (1981) reported molecular masses of between 30,000 and 40,000 for Bacteroides bivius and Bacteroides asaccharolyticus.

Isoelectric Focusing

Beta-lactamases from the Bacteroides focus in the acid range. A single pattern of bands consisting of a main band and several secondary bands is usually produced. There is evidence that species-specific beta-lactamases are produced by the members of the Bacteroides fragilis group (Leung and Williams, 1978; Nord and Olsson-Liljequist, 1981; Timewell et al., 1981a and b).

Most of the beta-lactamases from Bacteroides fragilis produce a main band which focuses at an isoelectric point (pI) of 4.9 (Olsson et al., 1976; Leung and Williams, 1978; Dornbusch et al., 1980; Timewell et al., 1981a; Maskell et al., 1984; Eley and Greenwood, 1986b). However, pIs between 4.2 and 5.6 have been reported (Olsson et al., 1976; Tally et al., 1979; Timewell et al., 1981; Simpson et al., 1982; Eley and Greenwood, 1986a and b). Enzymes from the remainder of the Bacteroides fragilis group focus between 4.0 and 5.6 (Leung and Williams, 1978; Tally et al., 1979; Dornbusch et al., 1980; Olsson-Liljequist et al., 1980; Timewell et al., 1981a; Maskell et al., 1984; Eley and Greenwood, 1986a).

Variation in the reported pIs may be due to the presence of more than one type of beta-lactamase (Phillips and Shannon, 1982; Simpson et al., 1982), or to different pIs being reported for the same enzyme (Phillips and Shannon, 1982). Phillips and Shannon (1982) suggest the use of standard enzyme preparations, reference to which should standardise results.

Cell Membrane Permeability Barriers

Gram-negative bacteria have a complex outer cell envelope with pores through which molecules with appropriate charge and shape, weighing less than 600 daltons can pass (Fig 1.10). Alterations in cell permeability can result in reduced susceptibility to all antibiotics which use the hydrophilic pathway to gain access to the periplasm (Livermore and Pitt, 1986). Work with permeability mutants of Pseudomonas and E. coli has provided direct evidence that the cell envelope of Gram-negative bacteria can effectively exclude beta-

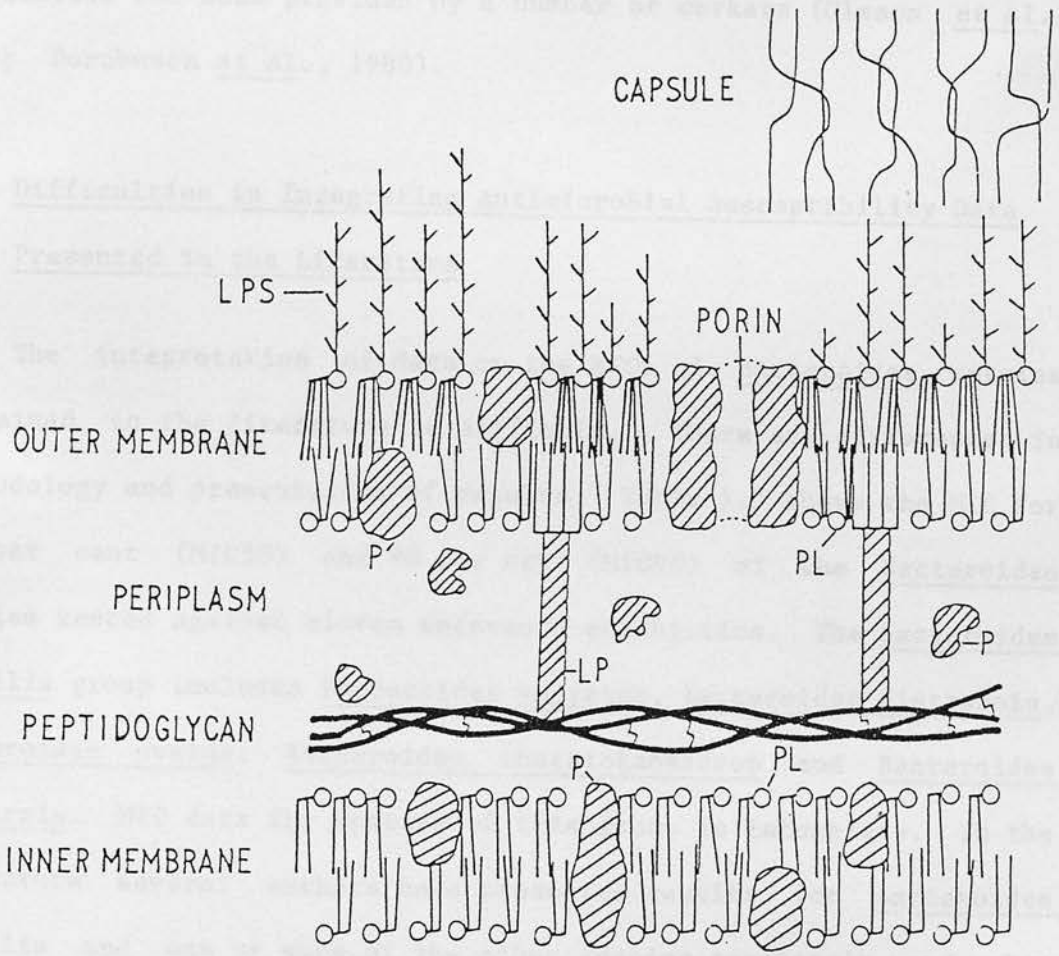


Figure 1.10

Structure of the cell envelope of a typical Gram-negative bacterium. (from Hancock and Poxton, 1988)

lactam antibiotics from their target enzymes (Richmond et al., 1976; Zimmermann and Rosselet, 1977; Zimmermann, 1980; Godfrey et al., 1984). Indirect evidence on the importance of membrane impermeability in the resistance of Bacteroides to beta-lactamase antibiotics has been provided by a number of workers (Olsson et al., 1979; Dornbusch et al., 1980).

2.6 Difficulties in Integrating Antimicrobial Susceptibility Data Presented in the Literature

The intepretation of data on the MICs of Bacteroides species contained in the literature is difficult. There are differences in methodology and presentation of results. Table 1.3 shows the MIC for 50 per cent (MIC50) and 90 per cent (MIC90) of the Bacteroides species tested against eleven reference antibiotics. The Bacteroides fragilis group includes Bacteroides vulgatus, Bacteroides distasonis, Bacteroides ovatus, Bacteroides thetaiotaomicron and Bacteroides uniformis. MIC data for species of this group is incomplete. In the literature several authors have presented results for Bacteroides fragilis and one or more of the other species separately and then grouped the rest as the Bacteroides fragilis group.

The literature values for the achievable serum levels (maximum concentration obtainable in serum after normal dosages) of antibiotics vary. The concentrations presented in Table 1.3 are according to Watt (1979), Rolfe and Finegold (1981) and Queener et al. (1986). Despite the fact that the achievable serum level for penicillin is 20 mg/l (Watt, 1979) several authors have interpreted MIC values of >2-4 mg/l as indicative of resistance to this

TABLE 1.3

The Minimum Inhibitory Concentration[1] of Eleven Reference Antibiotics to Bacteroides Species.*

(a) Bacteroides fragilis

Antibiotic		Number of Isolates	Geometric mean MIC	
			MIC50 (mg/1)	MIC90 (mg/1)
Chloramphenicol	(16)[2]	153	<5.3	7.3
Clindamycin	(8)	504	0.26	1.6
Erythromycin	(3)	122	>6.2<12.5	>25.0
Metronidazole	(16)	323	0.67	1.1
Imipenem	(32)	189	0.18	0.60
Moxalactam	(32)	244	1.3	8.8
Cefoxitin	(32)	968	<8.0	<22.9
Cefotetan	(32)	278	7.0	38.0
Cefotaxime	(16)	165	<13.1	<60.9
Cefuroxime	(32)	55	8.0	64.0
Penicillin G	(20)	198	<15.0	<30.1

1 Results prepared from references cited in the text.

2 Peak serum levels mg/1.

*These data should be interpreted with caution as they represent the findings of many different workers with non-standardised methods. The serum levels indicated by various authors are open to various interpretations and the original references should be consulted.

TABLE 1.3 (continued)

(b) Bacteroides thetaiotaiomicron

Antibiotic		Number of Isolates	Geometric mean MIC	
			MIC50 (mg/1)	MIC90 (mg/1)
Chloramphenicol	(16)[1]	10	6.3	6.3
Clindamycin	(8)	69	1.1	6.6
Erythromycin	(3)	ND[2]	ND	ND
Metronidazole	(16)	69	1.0	2.0
Imipenem	(32)	13	0.5	1.0
Moxalactam	(32)	45	25.4	50.8
Cefoxitin	(32)	85	18.8	38.7
Cefotetan	(32)	47	40.3	>64.0
Cefotaxime	(16)	55	64.0	>128.0
Cefuroxime	(32)	13	64.0	>128.0
Penicillin G	(20)	33	22.6	32.0

1 Peak serum levels mg/1.

2 ND = Not determined.

TABLE 1.3 (continued)

(c) Bacteroides distasonis

Antibiotic		Number of Isolates	Geometric mean MIC	
			MIC50 (mg/1)	MIC90 (mg/1)
Chloramphenicol	(16)[1]	ND[2]	ND	ND
Clindamycin	(8)	44	1.0	2.0
Erythromycin	(3)	ND	ND	ND
Metronidazole	(16)	34	0.63	2.0
Imipenem	(32)	19	0.5	2.0
Moxalactam	(32)	33	11.3	64.0
Cefoxitin	(32)	47	20.2	53.8
Cefotetan	(32)	28	32.0	>45.3
Cefotaxime	(16)	23	11.3	64.0
Cefuroxime	(32)	ND	ND	ND
Penicillin G	(20)	14	16.0	2.0

1 Peak serum levels mg/1.

2 ND = Not determined.

TABLE 1.3 (continued)

(d) Bacteroides ovatus

Antibiotic		Number of Isolates	Geometric mean MIC	
			MIC50 (mg/l)	MIC90 (mg/l)
Chloramphenicol	(16)[1]	ND[2]	ND	ND
Clindamycin	(8)	20	0.63	1.6
Erythromycin	(3)	ND	ND	ND
Metronidazole	(16)	20	0.25	1.0
Imipenem	(32)	ND	ND	ND
Moxalactam	(32)	19	ND	ND
Cefoxitin	(32)	23	20.0	40.0
Cefotetan	(32)	20	>32.0	>32.0
Cefotaxime	(16)	19	64.0	128.0
Cefuroxime	(32)	ND	ND	ND
Penicillin G	(20)	16	ND	ND

1 Peak serum levels mg/l.

2 ND = Not determined.

TABLE 1.3 (continued)

(e) Bacteroides vulgatus

Antibiotic		Number of Isolates	Geometric mean MIC	
			MIC50 (mg/1)	MIC90 (mg/1)
Chloramphenicol	(16)[1]	ND[2]	ND	ND
Clindamycin	(8)	10	0.008	0.12
Erythromycin	(3)	ND	0.12	2.0
Metronidazole	(16)	10	0.12	2.0
Imipenem	(32)	ND	ND	ND
Moxalactam	(32)	11	0.25	0.5
Cefoxitin	(32)	11	0.5	1.0
Cefotetan	(32)	10	0.25	2.0
Cefotaxime	(16)	11	0.12	16.0
Cefuroxime	(32)	ND	ND	ND
Penicillin G	(20)	10	1.0	2.0

1 Peak serum levels mg/1.

2 ND = Not determined.

TABLE 1.3 (continued)

(f) Bacteroides fragilis group[1]

Antibiotic		Number of Isolates	Geometric mean MIC	
			MIC50 (mg/l)	MIC90 (mg/l)
Chloramphenicol	(16)[2]	173	<6.0	7.5
Clindamycin	(8)	381	<0.30	1.4
Erythromycin	(3)	34	>4<8	>16<32
Metronidazole	(16)	177	0.45	1.6
Imipenem	(32)	133	0.3	0.88
Moxalactam	(32)	155	2.6	12.8
Cefoxitin	(32)	416	<5.8	20.0
Cefotetan	(32)	70	9.2	>24.7
Cefotaxime	(16)	15	<7.1	48.0
Cefuroxime	(32)	8	20.2	64.0
Penicillin G	(20)	28	<6.5	18.1

1 Includes ALL species of this group.

2 Peak serum levels mg/l.

TABLE 1.3 (continued)

(g) Bacteroides melaninogenicus/intermedius/asaccharolyticus

Antibiotic		Number of Isolates	Geometric mean MIC	
			MIC50 (mg/l)	MIC90 (mg/l)
Chloramphenicol	(16)[1]	77	0.95	2.2
Clindamycin	(8)	138	<0.30	0.13
Erythromycin	(3)	9	>0.1<0.5	0.5
Metronidazole	(16)	122	<0.35	1.6
Imipenem	(32)	52	0.03	0.16
Moxalactam	(32)	87	0.5	9.2
Cefoxitin	(32)	153	0.65	4.2
Cefotetan	(32)	73	0.79	2.0
Cefotaxime	(16)	70	0.49	10.1
Cefuroxime	(32)	ND[2]	ND	ND
Penicillin G	(20)	39	1.4	22.6

1 Peak serum levels mg/l.

2 ND = Not determined.

TABLE 1.3 (continued)

(h) Bacteroides bivius/disiens

Antibiotic		Number of Isolates	Geometric mean MIC	
			MIC50 (mg/l)	MIC90 (mg/l)
Chloramphenicol	(16)[1]	76	1.9	4.0
Clindamycin	(8)	103	0.07	0.08
Erythromycin	(3)	ND[2]	ND	ND
Metronidazole	(16)	95	1.3	2.0
Imipenem	(32)	31	0.03	0.06
Moxalactam	(32)	31	2.0	11.3
Cefoxitin	(32)	103	<1.1	<6.6
Cefotetan	(32)	32	5.6	16.0
Cefotaxime	(16)	42	1.6	11.3
Cefuroxime	(32)	ND	ND	ND
Penicillin G	(20)	57	5.7	<32.0

1 Peak serum levels mg/l.

2 ND = Not determined.

TABLE 1.3 (continued)

(i) Bacteroides species*

Antibiotic		Number of Isolates	Geometric mean MIC	
			MIC50 (mg/l)	MIC90 (mg/l)
Chloramphenicol	(16)[1]	67	<2.7	>3.7
Clindamycin	(8)	121	<0.21	1.2
Erythromycin	(3)	50	>0.5<1	>2<4
Metronidazole	(16)	60	<0.66	>2.0
Imipenem	(32)	42	0.13	5.1
Moxalactam	(32)	42	1.0	>64
Cefoxitin	(32)	121	<2.7	26.1
Cefotetan	(32)	41	5.0	26.5
Cefotaxime	(16)	30	1.4	256.0
Cefuroxime	(32)	ND[2]	ND	ND
Penicillin G	(20)	11	1.0	20.0

1 Peak serum levels mg/l.

2 ND = Not determined.

*These data were obtained from reports in which Bacteroides species were unclassified and did not belong to the groups recorded earlier in this table.

antibiotic (Brown and Waatti, 1980). In this review resistance to penicillin is interpreted as MIC values of >20 mg/l.

2.7 Comparative Efficacy of Antibiotics Used in the Treatment of Bacteroides Infections

All the non-beta-lactam antibiotics except erythromycin have been shown to be active against the Bacteroides (Table 1.3). Clindamycin, chloramphenicol, metronidazole and imipenem showed consistently high activity against all species. Results in the literature indicate that these antibiotics are the most effective of all the reference antibiotics for the treatment of Bacteroides infections.

Members of the Bacteroides fragilis group possess intrinsic resistance to penicillin (Tally et al., 1979; Acar et al., 1981). However, since most beta-lactamase enzymes produced by this group have minimal penicillinase activity resistance is possibly due to another resistance mechanism. Resistance in Bacteroides of the melaninogenicus group (Bacteroides bivius, disiens, intermedius and asaccharolyticus) is due in part to the production of penicillinase enzymes. Of the reference antibiotics (antibiotics with activity against Bacteroides tested during this study) penicillin was the least active with this group.

The newer cephalosporins (of the second and third generation) have been reported to have broader spectra of antimicrobial activity than the older agents and to be more resistant to hydrolysis by beta-lactamases (Fu and Neu, 1978; Jorgensen et al., 1980; Collatz

et al., 1984). Despite their greater resistance to the beta-lactamases of aerobes, cefotaxime and cefuroxime are still susceptible to the beta-lactamases of the Bacteroides fragilis group (O'Callaghan et al., 1976; King et al., 1980; Rolfe and Finegold, 1981). Relative hydrolysis rates of 58 and 73 per cent for cefuroxime and 28 to 54 per cent for cefotaxime have been reported by King et al. (1980), Schrinner et al. (1980) and Fu and Neu (1981). These rates indicate that cefotaxime should be more active than cefuroxime against the Bacteroides fragilis group. The results in table 1.3 show that their activities are similar and cefuroxime is slightly more active with some species. Resistance to beta-lactamase is therefore not the only mechanism of resistance to the cephalosporins.

There is variation in the literature regarding the relative activities of the cefamycins. In general the activities of cefoxitin and cefotetan are more similar to each other than to that of moxalactam. All cefamycins have been shown to be inhibitors of beta-lactamase activity (Olsson et al., 1976; Darland and Birnbaum, 1977). Their activity is therefore independent of inoculum size (Fass, 1979; King et al., 1980) and the presence of beta-lactam inhibitors (Wise et al., 1982; Piddock and Wise, 1987).

Moxalactam has been shown to be superior to cefoxitin in beta-lactamase inhibition (Fu and Neu, 1981). It has been suggested that this may be due to the presence of the carboxyl group at position 10 (Fig 1.7) since this group appears to confer similar beta-lactamase stability and inhibitory activity to carbenicillin (Fu and Neu, 1981). The increased beta-lactamase inhibition may be responsible

for the increased activity of moxalactam, as compared to cefoxitin, against some species of the Bacteroides fragilis group.

Cefoxitin shows greater activity against Bacteroides thetaiotaomicron than moxalactam. Evidence that this was due to the differential susceptibility of, or access to the relevant penicillin binding proteins was provided by Eley and Greenwood (1984). Recently novel beta-lactamases conferring resistance to the cefamycins have been found in Bacteroides fragilis (Cuchural et al., 1983; Yotsuji et al., 1983; Eley and Greenwood, 1986b). In most Bacteroides strains, however, the likely mechanism of resistance is reduced drug uptake or reduced affinity of drugs for the target enzymes.

Imipenem is the most active beta-lactam drug against Bacteroides. Studies have shown it to be resistant to degradation by the beta-lactamases of these species (Borobio et al., 1981; Hanslo et al., 1981; Richmond, 1981; Rolfe and Finegold, 1981; Tally and Jacobus, 1983). There is moderate affinity between the enzymes and the drug but no detectable hydrolysis occurs (Labia et al., 1986).

Imipenem has been shown to be highly efficient at penetrating Gram-negative cells (Williams et al., 1986). It may therefore retain inhibitory activity against less permeable organisms. Piddock and Wise (1986) reported that compounds that bound to PBP 1 kill more rapidly than compounds that bound to PBP 2 and 3. They noted that beta-lactamase compounds that kill quickly by binding to PBP 1 are most effective in antimicrobial susceptibility studies in vitro. Imipenem and the cefamycins have been shown to fall into this category (Nolan and Jude, 1983; Piddock and Wise, 1986).

2.8 Bacteroides Species Variation in Antimicrobial Susceptibility Patterns

Speciation of the Bacteroides fragilis group is necessary before determination of the MICs (Rolfe and Finegold, 1981; Eley and Greenwood, 1984). Table 1.3 shows that the antibiotic susceptibility patterns differ within this group. In general Bacteroides thetaiotaomicron and Bacteroides distasonis are the most resistant, while Bacteroides vulgatus is the most sensitive.

The relative activities of the reference antibiotics also differ between the species. The overall trends in antimicrobial susceptibility for the group as a whole is therefore not necessarily representative of the trends within a particular species. Within the non-fragilis group there are distinct patterns of antimicrobial susceptibility which differ from those of the members of the fragilis group.

D. ANTIBIOTIC RESISTANCE TRANSFER IN BACTEROIDES

1. Possible Significance of Antibiotic Resistance in Bacteroides

It has been noted that the genus Bacteroides may represent a significant repository of clinically important plasmids (Stiffler et al., 1974; Guiney and Davis, 1975; Del Bene et al., 1976; Tinnell and Macrina, 1976; Shoemaker and Salyers, 1987). In the colon the Bacteroides are in intimate contact with facultative, Gram-negative rods such as E. coli, in which the occurrence and importance of transferable drug resistance has been well documented (Goebel

et al., 1974; Gyles et al., 1974; Macrina et al., 1974; Thayer, 1979; Takahashi and Nagano, 1984). No function has been established for most of the plasmids isolated from Bacteroides and they have therefore been classified as cryptic (Stiffler et al., 1974; Tinnell and Macrina, 1976; Wallace et al., 1981; Mays et al., 1982; Riley and Mee, 1984). Several attempts have been made to correlate the presence of plasmids with specific phenotypic properties which may be important in the expression of antibiotic resistance and virulence (Riley and Mee, 1984; Beul et al., 1985). A correlation has been established between antibiotic resistance and plasmid presence in Bacteroides.

1.1 Relationship between Plasmid Presence and Antibiotic Resistance in Bacteroides

The existence of Bacteroides plasmid-borne resistance to the macrolide-lincosamide group of antibiotics has been demonstrated. Tally et al. (1979, 1982) isolated a 25.6MDa plasmid designated pBFTM10 from Bacteroides fragilis TMP10 which conferred resistance to clindamycin and erythromycin. A plasmid designated pCPI which is similar to pBFTM10 was isolated by Guiney et al. (1984). Smith and Macrina (1984) isolated a 51.3MDa plasmid, pBI136 from Bacteroides fragilis IB106; and Rotimi et al. (1981) isolated a 46MDa plasmid from a strain of Bacteroides fragilis (M2178). All conferred resistance to clindamycin and erythromycin.

Plasmid-mediated resistance to the M-L antibiotics has been associated with resistance to other classes of antibiotics. A 27MDa

plasmid designated pBF4 (also called pIP410) isolated from Bacteroides fragilis V479-1 by Welch et al. (1979) confers resistance to clindamycin, erythromycin and tetracycline (Privatera et al., 1979; Welch and Macrina, 1981; Shoemaker et al., 1985). Rotimi et al. (1981) also isolated from a Bacteroides fragilis strain (M490) a plasmid of between 46 and 62MDa which conferred resistance to erythromycin, tetracycline and chloramphenicol. In addition, these authors (Rotimi et al., 1981) isolated a 38MDa plasmid which conferred resistance solely to tetracycline from a Bacteroides fragilis strain (W3139), and a 46MDa plasmid which conferred resistance to tetracycline and chloramphenicol from a strain of Bacteroides thetaiotaomicron (C4/3).

2. Methods of Antibiotic Resistance Transfer in Bacteroides

It is quite common for plasmids carrying genetic information to confer conjugal donor ability on the cells in which they reside (Tinnell and Macrina, 1976). This low frequency transfer is not impeded by anaerobic conditions and may be intrageneric or intergeneric (Stallions and Curtiss III, 1972; Guiney and Davis, 1978). The genetic basis for the spread of antibiotic resistance in Bacteroides species is not well understood. It is the subject of much current investigation. Antibiotic resistance transfer has been demonstrated in Bacteroides and can be plasmid or non-plasmid associated.

2.1 Plasmid-Associated Transfer of Antibiotic Resistance in Bacteroides

Plasmid-associated transfer has been demonstrated by several groups of workers (Mancini and Behme, 1977; Privatera et al., 1979; Tally et al., 1979, 1982; Welch et al., 1979; Young and Mayer, 1979; Rotimi et al., 1981; Welch and Macrina, 1981; Smith and Macrina, 1984; Martinez-Suarez, 1985). The process has been investigated.

2.1.1 Mechanism of Transfer

Genetic information can be transferred between bacteria by conjugation, transduction or transformation. The transfer process was insensitive to DNAase and this rules out transformation as the mode of genetic exchange. Donor cell filtrates or chloroform-treated donor cell supernatants were not able to mediate resistance transfer so that a transduction mechanism is unlikely. In addition, close cell-to-cell contact appeared to be necessary (Privatera et al., 1979; Tally et al., 1979, 1982; Welch et al., 1979; Rotimi et al., 1981). These observations have led to the conclusion that the process of transfer is by conjugation.

Successful transfers were obtained by the use of filter mating techniques (Welch et al., 1979; Tally et al., 1979; Rotimi et al., 1981), by the mixing of broth cultures on agar surfaces (Privatera et al., 1979), or by centrifugation techniques (Rotimi et al., 1981). Rotimi et al. (1981) were unable to demonstrate transfer in simple mixed liquid cultures. They argued that the formation of stable

mating pairs may require external support which would be necessary if the sex pili were particularly fragile or if conjugation required an alteration of the cell surfaces.

2.1.2 Frequency of Transfer

The frequency of transfer per donor cell varied between 10^{-2} and 10^{-9}. Welch *et al*. (1979) observed greatly increased frequency of transfer in intraspecies matings as compared with interspecies mating. Similar results were obtained by Rotimi *et al*. (1981). Welch *et al*. (1979) concluded that this indicated the existence of a host restriction-modification system in *Bacteroides*. This could be the factor responsible for the failure of Rotimi *et al*. (1981) to transfer resistance plasmids from *Bacteroides fragilis* to *Bacteroides melaninogenicus* and *Bacteroides asaccharolyticus*.

However, Tally *et al*. (1979) noted that only certain transcipients were able to transfer at high frequency. They observed a similar variation in transfer frequencies (between 10^{-2} and 10^{-9}) during plasmid transfer between strains of *Bacteroides fragilis*. A possible explanation is the postulate of Welch *et al*. (1979) that there may be interstrain differences in surface receptors needed for plasmid transmission or differences in the ability of plasmids to replicate or be expressed in a given host strain.

2.1.3 Plasmid Characterisation

Four self-transmissible conjugative plasmids which confer MLS resistance in *Bacteroides* have been extensively studied (Welch and

Macrina, 1981; Tally et al., 1982; Guiney et al., 1984; Smith, 1985). Restriction endonuclease mapping of pBF4 (also called pIP410) and pBFTM10 have localised the MLS resistance determinant (MLS^r) to a 3.8 and 4.0Kb EcoRI-D fragment respectively. DNA homology studies using Southern blots have demonstrated homology between these fragments as well as the 4.0Kb EcoRI-B fragment of pCP1 (plasmid similar to pBFTM10) implicated in clindamycin resistance (Welch and Macrina, 1981; Shimell et al., 1982; Tally et al., 1982; Guiney et al., 1984).

The MLS^r determinants on these plasmids hybridise with a 10.1Kb segment of pBI136. However, significant differences occur in the size and restriction enzymes recognition sites as well as the genetic properties of this segment (Smith, 1985). The homologous sequences on pBI136 are found on a 7.2Kb EcoRI-C and a 2.9 EcoRI-G fragment. Neither of these fragments express tetracycline resistance when cloned into E. coli as has been demonstrated with the EcoRI-D fragment from pBF4 and the EcoRI-B fragment from pCP1 (Guiney et al., 1984; Smith, 1985).

The region of pBI136 implicated in MLS resistance resides on an 8.4Kb fragment which consists of two directly repeated sequences (DRS) of 1.2Kb and an intervening sequence of 6.0Kb (Smith, 1985). A DRS has been shown to flank both sides of the MLS resistance determinant of pBF4 and pBFTM10 (Shimell et al., 1982; Guiney et al., 1984). This DRS is a 0.5-1Kb segment of DNA and has been shown to be common to all four plasmids (Smith, 1985; Rasmussen et al., 1987). The plasmids vary in size and restriction endonuclease cleavage pattern. With the exception of the MLS^r

determinants they bear no detectable homology (Shimell et al., 1982; Marsh et al., 1983; Guiney et al., 1984; Smith, 1985).

The Host Range of Antibiotic Resistance Plasmids

Early work with resistance plasmids in Bacteroides indicated that the conjugative host range was limited to the Bacteroides fragilis group (Del Bene et al., 1976; Privatera et al., 1979; Welch et al., 1979). The work of Anderson (1979) presented evidence that inhibition of conjugation of E. coli with Bacteroides was due to the suppression of growth of E. coli in mixed culture. Three groups of workers reported plasmid-mediated transfer of antibiotic resistance from Bacteroides to E. coli (Mancini and Behme, 1977; Young and Mayer, 1979; Rotimi et al., 1981) but the plasmids remained uncharacterised. (The report of Guiney and Davis [1979] on Bacteroides to E. coli plasmid transfer is no longer relevant as Bacteroides ochraceus has been renamed Capnocytophaga ochraceus.) Similarly, successful transfer of antibiotic resistance from E. coli to Bacteroides had been reported by Burt and Woods (1976). Heat treatment of the anaerobes by these workers was thought to have inhibited restriction systems and allowed tranfer but the genetic elements involved were not identified.

However, more recent work has shown that the MLS^r determinant and the flanking DR sequences on pBF4, pBFTM10 and pBI136 undergo transposition in both E. coli and Bacteroides species. They have been designated transposons Tn4351, Tn4400 and Tn4551 respectively

(Robillard et al., 1985; Shoemaker et al., 1985; Smith and Spiegel, 1987). Tn4351 has been the most extensively studied.

Rasmussen et al. (1986) investigated the nucleotide sequence of the MLS^r gene of Tn4351 which they designated ermF. They demonstrated similarities between the amino acid composition from this gene and those of erm genes isolated from six Gram-positive bacteria. These results supported the hypothesis that resistance mechanisms in pathogenic bacteria may have originated in antibiotic-producing microorganisms (Walker and Walker, 1970; Benveniste and Davies, 1973).

The DR sequences which flank the MLS^r genes of Tn4351 and Tn4400 have been shown to be insertion sequences (Robillard et al., 1985; Ras et al., 1987). Evidence that the same is true for the DRS of Tn4551 has been presented by Smith and Spiegel (1987). The upstream insertion sequence of ermF which promotes transposition of Tn4351 in E. coli has been designated IS4351 (Rasmussen et al., 1987). The moles percentages G + C content of IS4351 was shown by these workers to be similar to that of Bacteroides chromosomal DNA. In addition, they found copies of this sequence in the chromosome of a strain of Bacteroides fragilis. DNA homology has been demonstrated between IS4351 and the DR sequences of Tn4400 and Tn4451 (Shimell et al., 1982; Smith and Gonda, 1985). These results led to the postulate by Rasmussen et al. (1987) that the insertion sequences of the three transposons are of Bacteroides origin, similarity being due to the widespread dissemination of a single element on Bacteroides plasmids.

2.1.5 Expression of Antibiotic Resistance Phenotypes

Several groups of workers have shown that antibiotic resistance genes which confer resistance on Bacteroides species do not confer resistance on E. coli and vice versa. The Cc^rErm^r determinants from the MLS resistance plasmids pBF4 and pBFTM10 are not conferred to susceptible strains of E. coli. In addition, a tetracycline resistance determinant designated $*Tc^r$ which resides on these plasmids is expressed in E. coli but not in Bacteroides (Guiney et al., 1984; Robillard et al., 1985; Shoemaker et al., 1985; Shoemaker et al., 1986). To date there are no known antibiotic resistance determinants which are expressed in both E. coli and Bacteroides species.

The evidence suggests that these phenotypic differences are due to differences in plasmid replication and gene expression. Broad host range IncP and IncQ plasmids from E. coli were shown to be unable to replicate in Bacteroides. Similarly, naturally occurring Bacteroides plasmids were unable to replicate in E. coli (Guiney et al., 1984; Shoemaker et al., 1985, 1986a, 1986b). A relationship between gene expression and growth conditions was established by Guiney et al. (1984). They showed that expression of the $*Tc^r$ determinant was dependent upon growth in the presence of oxygen.

2.2 Non-Plasmid Associated Transfer of Antibiotic Resistance in Bacteroides

Non-plasmid associated transfer of clindamycin and tetracycline resistance has been reported in Bacteroides (Macrina et al., 1981;

Mays et al., 1982). Resistance was transferred from a strain of Bacteroides fragilis (V503), which had a 3.7Md plasmid (pVA503), to a plasmidless strain of Bacteroides uniformis (V528). The clindamycin resistance determinant of the conjugative plasmid pBF4 shared homology with pVA503. However, the transfer of drug resistance seemed independent of the transfer of pVA503. Transfer occurred by a conjugation-like process and the location of the determinants may be chromosomal. Non-plasmid-mediated transfer of resistance to clindamycin and tetracycline was also reported by Guiney et al. (1983). Homology was demonstrated between a 4.8Md EcoRI fragment of pBF4 and the whole-cell DNA of the transfer proficient Bacteroides fragilis strain. Transfer of tetracycline resistance in the absence of plasmid DNA was reported by Privatera et al. (1981) and Rashtchian et al. (1982).

Several attempts to demonstrate transferable resistance to beta-lactam antibiotics have proved unsuccessful (Anderson and Sykes, 1973; Del Bene et al., 1979; Olsson-Liljequist et al., 1980). However, Rashtchian et al. (1982) successfully transferred cefoxitin resistance from a multiple plasmid-containing strain of Bacteroides thetaiotamicron to other Bacteroides strains from the Bacteroides fragilis group. Plasmid DNA was not detected in all cefoxitin-resistant transconjugants. This indicates a non-plasmid transfer mechanism or the presence of an episome which may integrate into the chromosome.

E. Virulence Mechanisms in Bacteroides

The ability of a pathogen to establish itself in a host is dependent on it overcoming the natural defence mechanisms of the host which would prevent colonization and multiplication. The attachment of bacteria to mucosal epithelial cells is a prerequisite for the establishment of indigenous flora as well as pathogens. Pilus-like threads and capsular polysaccharide have been implicated in the Bacteroides (Onderdonk et al., 1977; Babb and Cummins, 1978; Burt et al., 1978; Woo et al., 1979; Lambe et al., 1984; Riley and Mee, 1984; Patrick et al., 1986).

Capsules have been associated with the virulence of a number of bacteria (Robbins et al., 1980). The capsule of some members of the Bacteroides fragilis group has been identified as a possible virulence factor. Several groups of workers have demonstrated increased resistance to phagocytosis and opsonophagocytic killing by neutrophils in encapsulated strains of Bacteroides fragilis (Connolly et al., 1984; Riley and Mee, 1984; Patrick et al., 1984).

The capsular polysaccharide of Bacteroides fragilis has been shown to potentiate abscess production (Onderdonk et al., 1977) while that of Bacteroides thetaiotaomicron has been shown to confer resistance to a strain-specific phage (Burt et al., 1978). An unidentified component of the outer membrane of the Bacteroides fragilis group is known to inhibit the chemotaxis of polymorphonuclear neutrophils towards the bacterial cells (Adamu and Sperry, 1981). Inhibition of phagocytosis has similarly been demonstrated in the melaninogenicus/oralis and asaccharolytic groups

(Ingham et al., 1978, 1981; Okuda and Takazoe, 1973; Socransky and Gibbons, 1965; Sundquist et al., 1979).

Pathogenic bacteria may produce enzymes that are able to cleave plasma proteins involved in the host defences (Muller and Werner, 1970; Werner and Muller, 1971; Carlsson et al., 1984; Sundquist et al., 1985). They may also release substances which cause consumption of serum complement (Sundquist et al., 1984, 1985). Sundquist et al. (1985) provided evidence that these mechanisms were responsible for the observed inhibition of phagocytosis. They also demonstrated degradation of host plasma protein inhibitors which may be active against bacterial cells; and the degradation of haem-binding plasma proteins to products which may support microbial growth. The production of catalase and superoxidedismutase, observed by Gregory et al. (1977) and Wilkins et al. (1978), may facilitate the survival of anaerobes in environments which contain oxygen.

CHAPTER 2

ISOLATION AND IDENTIFICATION OF *BACTEROIDES* FOR THE ESTABLISHMENT OF A *BACTEROIDES* CULTURE COLLECTION

INTRODUCTION

The acquisition of strains of Bacteroides was a prerequisite for subsequent investigations. Strains were collected from several sites. These included the female genital tract, blood cultures and wound infections. All strains were positively identified as Bacteroides.

There have been numerous reports describing the normal flora of the female genital tract (Bartlett et al., 1977; Brown, 1982; Duerden, 1980; Gorbach et al., 1973; Hill et al., 1985; Larsen and Galask, 1982; Lindler et al., 1978; Ohm and Galask, 1975; Osborne et al., 1979; Watt et al., 1981). The microflora of the cervix was investigated with the aim of drawing general conclusions on the distribution of Bacteroides species while providing strains for further study. The Anatube Type II anaerobic transport system (developed in Belgium) was assessed for efficiency of transportation of anaerobic samples.

MATERIALS AND METHODS

Source of Strains

Bacterial strains were obtained from numerous sources. Strains designated MW were clinical isolates of Bacteroides supplied by Dr Brian Watt of the City Hospital, Edinburgh. MH strains were isolated from specimens processed by the diagnostic laboratories of the Royal Infirmary of Edinburgh. They were mainly from vaginal swabs (both high and low) with a small proportion from wound infections and blood cultures. NCTC strains were obtained from the National Collection of Type Cultures, Colindale Avenue, London. Strains designated MPRL were obtained from the culture collection of the Microbial Pathogenicity Research Laboratory of the Bacteriology Department, University of Edinburgh. MG strains were isolated from the cervix.

Isolation of Bacterial Strains from the Cervix

Samples were collected from the cervix of women attending the colposcopy clinic at the Elsie Inglis Hospital in Edinburgh. The women were undergoing examination as a result of abnormal cervical smears. They had no record of infection although in some cases there were signs of inflammation. All sampling was done at the time of speculum examination with the aid of a colposcope directed microscope. The surface investigated was the transformation zone between the endo and ecto cervix. No contact was made with other surfaces. The Anatube Type II system for the collection and transportation of anaerobic samples was used (Figs 2.1 and 2.2). All samples were processed on the day within 2 h of collection.

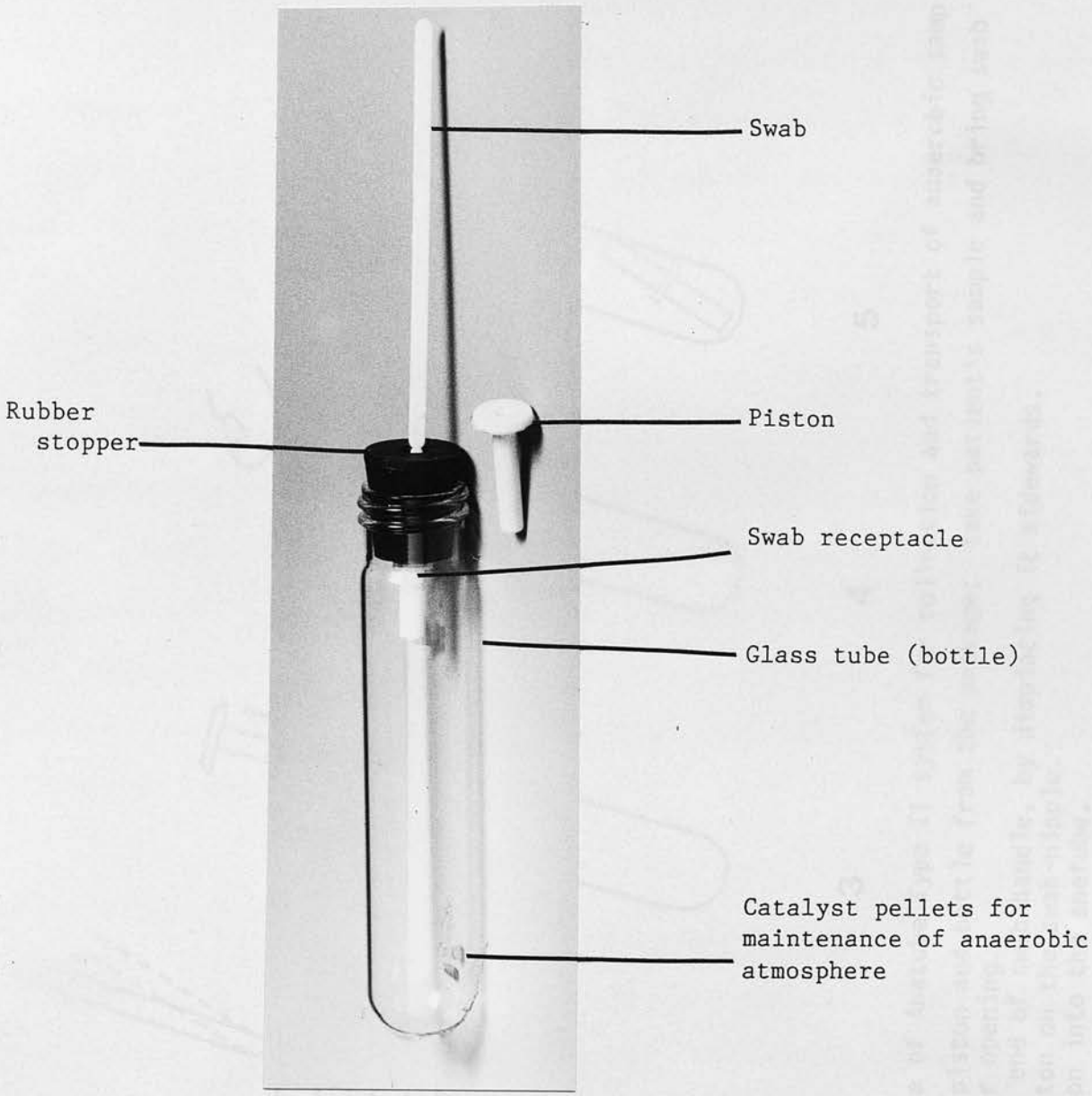


Figure 2.1

The Anatube Type II system for the collection and transport of anaerobic samples.

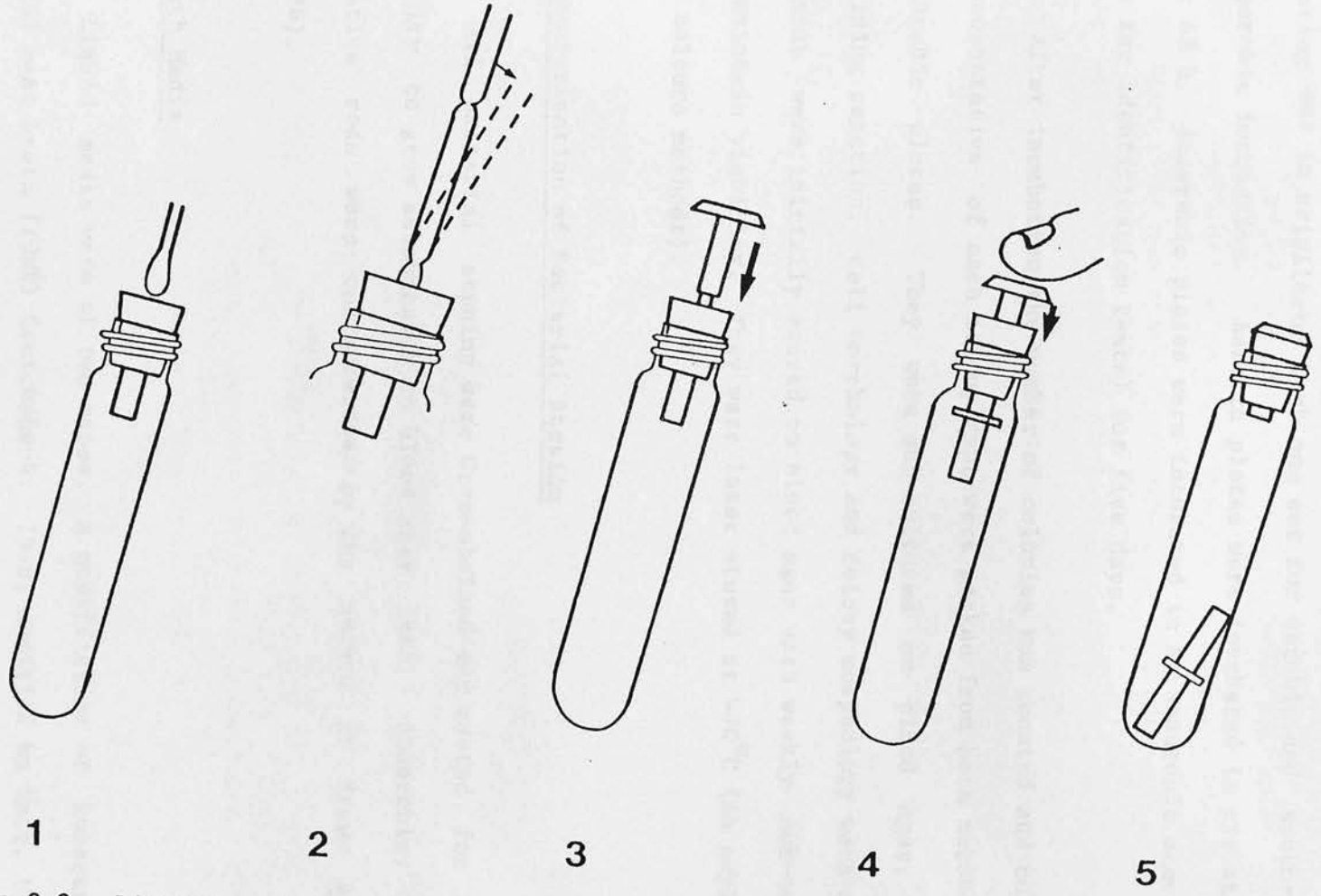


Figure 2.2 Directions for use of Anatube Type II system for collection and transport of anaerobic samples.

1. Remove swab, piston and bottle from the package; take patient's sample and bring swab in the stopper opening.
2. Break off the end of swab handle, by displacing it sidewards.
3. Place the piston on the swab-nipple.
4. Push the piston into the anatube.

Swabs were expressed in 1 ml pre-reduced diluent (saline or nutrient broth). Serial 10-fold dilutions (0.1 ml) up to 10^{-9} were spread-plated on to non-selective agar (combined blood agar as outlined for identification tests) and on Bacteroides selective agar (combined blood agar with kanamycin 75 mg/l and vancomycin 2.5 mg/l). Plating was in triplicate with one set for aerobic and another for anaerobic incubation. Aerobic plates were incubated in air at 37 °C for 48 h. Anaerobic plates were incubated in an anaerobic atmosphere (as for identification tests) for five days.

After incubation the number of colonies was counted and colonies representative of each colony type were picked from both aerobic and anaerobic plates. They were sub-cultured on blood agar. Gram staining reaction, cell morphology and colony morphology were noted. Strains were initially stored on blood agar with weekly sub-culture to maintain viability. They were later stored at -70°C (as explained for culture methods).

Characterisation of Bacterial Strains

All bacterial strains were Gram-stained and tested for their ability to grow aerobically on blood agar (BA). Anaerobic, Gram-negative rods were characterised by the method of Brown et al. (1988).

Growth Media

Liquid media were of two types, a modification of Robertson's cooked meat broth [(CMB) Cruickshank, 1968, modified by Watt, 1973)]

and proteose peptone yeast extract medium (PPY). The PPY contained 2% proteose peptone, 1% yeast extract and 0.5% sodium chloride. It was supplemented with final concentrations of haemin 5 mg/l, menadione 1 mg/l, cysteine hydrochloride 0.075% (w/v) and sodium carbonate 0.04% [(w/v) Deacon et al., 1978].

All solid media were prepared with Columbia agar base (Oxoid). Blood agar contained 5% outdated human blood or horse blood. Lysed blood agar (LBA) contained 5% saponin lysed blood, while combined blood agar (CBA) contained 4% whole blood and 1% freeze-thawed lysed blood. It was supplemented with menadione to a final concentration of 1 mg/l. Egg yolk agar (EYA) contained 0.25% sodium succinate, 0.25% yeast extract and 5% (v/v) egg yolk emulsion. It was supplemented with menadione and haemin to final concentrations of 1 g/l and 5 mg/l respectively (Duerden et al., 1976). Solid media for tolerance tests were prepared in the same way as EYA with the egg yolk being replaced with dye solution or rehydrated bile of the required concentration.

Culture Methods

Stock cultures were prepared by the modified method of White and Sands (1985). Strains were grown in 8 - 10 ml PPY for 24 - 72 h and the cells pelleted by centrifugation at 4000 g for 20 min in a bench centrifuge. Cells were resuspended in 1 ml pre-reduced PPY containing 10% (v/v) glycerol. The suspension was then used to coat sterile glass beads in cryogenic tubes with excess fluid being removed and discarded. Culture-coated beads were stored at -70°C. Before sub-culture cryogenic tubes were removed and placed in a

paraffin block which had also been stored at -70°C (to prevent thawing of unrequired beads). Individual beads were then aseptically removed as required into pre-reduced broth media while the paraffin block and beads for continued storage were returned to -70°C storage facility.

For identification tests the inoculum was a single bead. This was sub-cultured directly into 20 ml PPY or in the case of more fastidious strains, into 1 ml CMB which then served as the inoculum for the 20 ml PPY. Broth cultures were incubated for 24 - 48 h or until there was visible growth. Cultures of fast-growing strains that gave very turbid growth after 24 h incubation were diluted 1:10 in PPY before use. Incubation was at 37°C in an atmosphere of H_2 90% and CO_2 10% in anaerobic jars (Collee et al., 1972) or of H_2 10%, CO_2 10% and 80% in an anaerobic cabinet (Forma Systems Anaerobic Work Station).

Identification Tests

Identification was in two stages. Preliminary tests (Table 2.1) separated isolates into one of four groups, the Bacteroides fragilis group, the Bacteroides melaninogenicus/oralis group, the asaccharolytic Bacteroides and the Fusobacterium/Leptotrichia buccalis group. This was followed by secondary tests (Table 2.2) to determine the species of each strain. Gas-liquid chromatography was used in the identification of Fusobacterium strains and where necessary to confirm the identity of unreactive asaccharolytic strains.

TABLE 2.1

Primary Tests[1] for the Identification of Anaerobic Gram-negative Rods

Test	Pattern of results obtained with strains of			
	Bacteroides fragilis group	Bacteroides melaninogenicus /oralis group	Asaccharolytic Bacteroides group	Fusobacterium/ Leptotrichia buccalis group
Antibiotic resistance				
Neomycin 1000 ug/disk	R[2]	S	S/R	S
Kanamycin 1000 ug/disk	R	R	R (S)	S
Penicillin 2 i.u./disk	R	S/R	S	S
Tolerance				
Taurocholate 0.5%	+	I	I	+/I
Gentian violet 1/100,000	I	I	I (+)	+
Glucose fermentation	+	+	- (w)	+/-
Bile stimulation	+	I	I	+/I

1 Modified method of Duerden et al. (1980)

2 In antibiotic-susceptibility tests: R = resistant; S = sensitive; S/R = 30-70% of strains gave each result; in tolerance tests and bile stimulation: + = growth; I = inhibition; +/I = 30-70% of strains gave each result; I (+) = few species gave a positive result; in glucose fermentation: + = fermentation; +/- = 30-70% of strains ferment glucose; - (w) = a few strains weakly ferment glucose.

TABLE 2.2

Secondary Tests for the Identification of Anaerobic Gram-negative Rods

Bacteroides fragilis group	*Bacteroides melaninogenicus/ oralis* group	Asaccharolytic *Bacteroides* group	*Fusobacterium/ Leptotrichia buccalis* group
Indole	Indole	Indole	Indole
	Aesculin hydrolysis	Aesculin hydrolysis	Aesculin hydrolysis
Carbohydrate fermentation	Pigment	Pigment	Pigment
Sucrose		Gelatinase	
Rhamnose	Carbohydrate fermentation	Nitrate reduction	Carbohydrate fermentation
Trehalose	Lactose	Urease	Glucose
Xylan	Sucrose		Lactose
	Maltose		Sucrose
	Xylose		Mannose
	Cellobiose		Fructose
	Aesculin		Starch
		GLC	GLC

1 Method of Brown *et al.* (1988)

Gas-liquid Chromatography = GLC

Tests were performed in broth (tube tests) as well as on solid media (plate tests). Plate tests included antibiotic resistance tests, tolerance tests, pigment production and lipase production. In all cases the inoculum for these tests was 0.03 ml of PPY culture or dilution in PPY. This was spread over the surface of the plate or streaked out as required.

Tube tests were performed in Wasserman (WR) tubes (75 mm x 12 mm) containing 100 µl of appropriate liquid substrate, or a charcoal gelatin disk in CMB. The inoculum was 1 ml of culture or diluted culture. These tests included carbohydrate fermentation, nitrate reduction, urease production, gelatinase production and aesculin hydrolysis. The final concentration of substrates were as follows: fermentation tests, 1% (w/v) substrate; aesculin hydrolysis, 0.2% (w/v) aesculin; nitrate reduction, 200 mg/l potassium nitrate; urease production, 1% (w/v) urea.

Antibiotic Resistance Tests

Resistance to three antibiotics was tested by the disk-diffusion method. Disks containing neomycin 1000 ug, kanamycin 1000 ug and penicillin 2 international units were added to strains spread on to blood agar. After incubation* the diameters of zones of inhibition were measured. Strains were considered sensitive if the zone diameter was greater than 15 mm.

*24-72h

Pigment Production

The ability of strains to produce black or brown pigmented colonies was investigated on LBA and CBA. Streaked cultures were examined after incubation for 2 days with observations repeated daily for up to 7 days.

Lipase Production

Lipase production was tested on EYA. Strains were streaked out and examined daily up to four days for the formation of an irridescent layer around the colonies and development of restricted opacity in the medium.

Nitrate Reduction

The reduction of nitrate to nitrite was tested in the presence of potassium nitrate. Nitrite was detected by the production of a deep red colour on the addition of 0.25 ml of Nitrate Solution A and 0.25 ml of Nitrate Solution B (Sutter and Finegold, 1975).

Urease Production

Urease activity in the presence of urea was detected by an indicator (0.1% bromothymol blue pH 7.0) colour change. The raising of the pH by ammonia, the end-product of urea metabolism, resulted in a colour change from green to blue.

Gelatinase Production

The production of gelatinase was tested by the digestion of a charcoal-gelatin disk in CMB (Duerden et al., 1976). Incubation was for up to 2 weeks with observations at 1, 2, 7 and 14 days.

Aesculin Hydrolysis

Aesculin hydrolysis was tested in the presence of 0.2% aesculin. After incubation, a black discoloration in the presence of 0.25 ml of a 1% w/v aqueous solution of ferric ammonium citrate indicated hydrolysis.

Tolerance Tests

Each strain was streaked on to a series of three plates containing (i) no dye or bile salts (control), (ii) sodium taurocholate 0.5%, (iii) gentian violet 1 in 100,000. After incubation growth in the presence of the bile salts and sodium taurocholate was compared with growth on the control plate. Growth was considered inhibited if it was less than that on the control plate (as determined by visual inspection).

Bile Stimulation

Growth in PPY containing 1% (w/v) glucose (PPYG) was compared with growth in the presence of PPYG containing 2% (v/v) ox-bile.

Indole Production

Indole production was tested in PPY broth culture (1 ml) that was transferred to a sterile WR tube at the time of inoculation of the other identification tests. The presence of indole was detected by the development of a pink colour after extraction into toluene (0.25 ml, with shaking) and addition of Ehrlich's Reagent (0.25 ml).

Carbohydrate Fermentation

Growth in the presence of carbohydrates was compared to that of growth in a carbohydrate-free control. Culture turbidity was recorded on a scale of trace (growth just visible) and 1 to 4. Increases or decreases in turbidity relative to the control were noted as a possible indication of fermentation.* 0.1% bromothymol blue indicator (pH 7) was used to determine acid production due to fermentation. The test was considered positive for fermentation when the colour of the indicator remained dark green in the control but turned yellow in the carbohydrate-containing culture. Where equivocal results were obtained the pH was read with a meter. Fermentation was considered positive if the pH of the test carbohydrate was > 0.5 pH units below the carbohydrate-free control culture.

Gas-Liquid Chromatography (GLC)

The fatty acid end products of metabolism of selected strains were analysed on a Supelco SP1220 (15%) and H_3PO_4 (1%) on 100 - 120 mesh Chromosorb 101 in 1.5 m x 4 mm $^{i}/_{d}$ glass columns in a Pye Unicam

*Caution: The pH change was regarded as the important measurement.

104 chromatograph fitted with dual FID detectors. The operating conditions were: oven temperature, 148°C isothermal; carrier gas (Oxygen Free Nitrogen) 35 ml/min; hydrogen, 35 ml/min; compressed air, 525 ml/min; attenuation 5 x 10^2.

Samples for analysis were prepared by the modified method of Deacon et al. (1978). Broth cultures (2 ml) in 1% glucose (w/v) in PPY (PPYG) were acidified with five drops (0.15 ml) of 50% (v/v) sulphuric acid and centrifuged at 1900 g for 15 min. The supernatant was decanted and stored at 4°C in Bijou bottles.

Acidified supernatant was used directly in the case of volatile fatty acids. Non-volatile fatty acids were first converted to their volatile methyl esters. Methanol (2 ml) and 50% (v/v) sulphuric acid (0.4 ml) were added to acidified supernatant (1 ml) and the mixture left to stand overnight at room temperature. Distilled water (1 ml) and chloroform (0.5 ml) were added and the contents of the tube mixed by inversion. The lower chloroform layer was used for analysis.

Before use the column was 'cleared' with water for the analysis of the volatile fatty acids and with chloroform for the analysis of the non-volatile fatty acids. Sterile acidified PPYG was passed through the column to enable correction of peak heights of the test samples. Combined acid standards were used to establish peak heights and relative retention times of fatty acids within the column: 0.01 M acetic, propionic, isobutyric, n-butyric, isovaleric, n-valeric, isocaproic and n-caproic acids for the volatile fatty acids, and the methyl esters of 0.04 M lactic, and 0.01 M succinic and phenylacetic acids for the non-volatile fatty acids.

Test samples were loaded and run for 10 min to obtain volatile fatty acid profiles, and 20 min for non-volatile fatty acid profiles. All loading was by injection of 1 µl quantities from a 5 µl (SGE) syringe. Identification of peaks corresponding to the different acids was made by measurement of the relative retention times using the formula:

$$\text{Acid concentration (umol/ml)} = \frac{\text{Peak height of test} - \text{Peak height of PPYG control}}{\text{Peak height of standard}} \times \text{Concentration of standard}$$

RESULTS

A total of 151 organisms collected from the various sources were identified to species level (Appendix 1). The distribution of the strains between the three Bacteroides groups is summarised in Table 2.3.

Isolation of Organisms from the Cervix

The micro-organisms recovered from individual cervical samples are shown in Table 2.4. The total count varied from 10^3 to 10^8 organisms per sample. All samples contained mixed populations of aerobic and anaerobic micro-organisms with the percentage of anaerobes varying from less than one per cent to greater than ninety nine per cent. Wide variation in the presence of anaerobic Gram-negative rods was also observed. They were present in 21 of 25 patients (84%) and were absent from four (16%). When present they accounted for more than fifteen per cent of the total flora in thirteen patients (61.9%) and as less than ten percent of the total flora in eight patients (38.1%).

Most of the cervical isolates were not identified to species level. A large number of slow-growing strains were lost during subculture and storage. Of twenty nine isolates identified to the species level, seven belonged to the B. fragilis group, three to the B. melaninogenicus/oralis group, and sixteen to the asaccharolytic Bacteroides. Three of the strains belonged to the genus

TABLE 2.3

Distribution of Bacterial Strains in *Bacteroides* groups

Bacteroides Group	Number in group (total 151)	Per cent of total
B. fragilis group	88	58.2
B. fragilis species	57	37.7
Other *B. fragilis* species	31	20.5
B. melaninogenicus/oralis group	41	27.2
Asaccharolytic group	22	14.6

TABLE 2.4

Microorganisms Recovered from Endocervical Swabs from Women Attending a Colposcopy Clinic *

Patient number	Total aerobes (strict and facultative	Total anaerobes (strict)	Percentage strict anaerobes	Percentage Gram-negative anaerobic rods
1	1.6×10^4_2	2.0×10^3_3	11.1	-
2	0.4×10^2_4	1.4×10^3_7	97.4	-
3	9.7×10^5_5	1.1×10^6_6	99.1	33.0
4	1.6×10^5_5	4.2×10^6_2	96.3	19.3
5	1.2×10^3_3	0.9×10^2_3	0.1	-
6	7.2×10^3_3	0.1×10^3_5	1.5	<1.0
7	9.5×10^3_2	3.9×10^5_6	96.5	78.9
8	9.1×10^2_7	3.4×10^6_6	99.9	4.4
9	1.2×10^5_5	4.4×10^8_8	27.8	15.0
10	3.4×10^3_3	6.3×10^5_5	99.9	15.2
11	2.9×10^5_5	6.3×10^6_6	99.6	2.3
12	5.9×10^6_6	5.2×10^7_7	89.8	28.9
13	2.0×10^6	7.8×10^3_3	97.5	22.2
14	<10	3.0×10^4_4	99.9	2.2
16	1.1×10^3_5	8.1×10^6_6	98.7	<1.0
17	8.9×10^5_7	4.0×10^6_7	81.9	49.9
18	2.7×10^7_3	9.5×10^7_8	78.0	5.9
19	6.3×10^3_6	1.3×10^8_7	99.9	99.2
20	5.0×10^6_3	7.7×10^7_8	93.9	86.5
21	6.0×10^3_6	1.2×10^8_8	99.9	20.0
22	2.0×10^6_2	1.9×10^6_6	99.0	86.4
23	1.6×10^2_4	8.4×10^6_7	99.9	<1.0
24	3.2×10^4_7	8.7×10^7_7	2.7	-
25	1.3×10^7	9.3×10^7	87.6	53.1

*The counts represent the number of bacteria recovered from a swab expressed into 1ml pre-reduced diluent (see page 66).

Fusobacterium. Members of the B. fragilis group were isolated from three patients (12%) while B. fragilis was isolated from only one patient.

DISCUSSION

Early studies of the genital tract led to Doderleins concept that the vaginal flora was essentially homogeneous and consisted mainly of lactobacilli. This theory remained widely accepted until the 1970's despite the isolation of anaerobes from the genital tract as early as the 1920's (Harris and Brown, 1929). The increased understanding of the complexity of this flora has been facilitated by the application of improved techniques for the isolation and identification of obligately anaerobic bacteria.

A major problem in the recovery of anaerobic bacteria has been improper collection and transporation of specimens. Many fastidious organisms are extremely sensitive to oxygen and exposure to air should therefore be minimised. Transport media should maintain both aerobic and anaerobic organisms. It should be non-selective, non-bactericidal and have a low redox potential and minimal susceptibility to oxidation (Syed et al., 1972). This requirement for an ideal system has led to the development of commercial transport systems (Chow et al., 1975).

The Belgian anaerobic transport system under evaluation was found to be efficient for the transportation of samples. The percentage of identifiable cultures of strict anaerobes was in some cases up to 99%, indicating good survival conditions. This system has been used in a large scale study of the anaerobic flora of the oral cavity of sheep in which excellent results are also being obtained (McCourtie and Poxton, personal communication).

The microbial population of the female genital tract is dependent on the hormonal, anatomical, physiological and immunological status of individuals (Bartlett et al., 1977; Brown, 1982; Golperud et al., 1976; Larsen, 1982; Thadepalli et al., 1978). It is impossible to state precise figures for the prevalence of any organism in the genital tract due to differences in methodology, of the reports in the literature.

The majority of investigations reported in the literature have concentrated on the microflora of the vagina. Differences in anatomy and physiology of the cervix and vagina result in distinctive microbial populations (Bartlett et al., 1978; Hill et al., 1985). The flora of the cervix is known to be complex with several species of varying oxygen tolerance (Golperud et al., 1976; Gorbach et al., 1973; Ohm and Galask, 1975; Thadepalli et al., 1978). This investigation has confirmed the multibacterial nature of the microflora of the endocervix.

Anaerobes form a significant proportion of the endogenous flora of the cervix (Gorbach et al., 1973; Ohm and Galask, 1975). Variation in their percentage composition, such as that observed within this study, has been shown to exist between individuals as well as within individuals sampled over a period of time (Golperud et al., 1976; Gorbach et al., 1973; Ohm and Galask, 1975). Gorbach et al. (1973) found mixed aerobic and anaerobic flora in 70% of individuals with Bacteroides being present in 57%. Sanders et al. found Bacteroides in 65% of healthy women. Ohm and Galask (1975) found anaerobic Gram-negative rods in 29% of women while 86% of their total sample population had mixed aerobic and anaerobic flora. All

patients examined during this study had mixed flora and 84% had strains of anaerobic, Gram-negative rods.

Despite the loss of anaerobes before the completion of identification procedures it was possible to infer that the majority of Bacteroides strains isolated from the cervix were not from the B. fragilis group. B. fragilis itself is only rarely isolated from the genital tract of healthy women (Bartlett et al., 1977, 1978; Larson, 1982; Levison et al., 1976; Duerden 1980). In this study it was isolated from only one individual. This patient was not recorded as showing signs of inflammation.

The percentage of B. fragilis group isolates from the cervix was found to be 17% and four per cent, respectively, by Gorbach et al. (1973) and Ohm et al. (1975). The results of this study (12%) are intermediate between the two. A significant number of women carry non-identifiable Bacteroides species in the vagina (Hill et al., 1980). Similar strains were isolated from the cervix. Fusobacterium strains, in agreement with the results of other workers, were isolated much less frequently than Bacteroides strains (Bartlett et al., 1977; Hill et al., 1985; Levison et al., 1977).

The final Bacteroides culture collection was composed of strains from all three Bacteroides groups. This was considered appropriate for representative studies of the genus.

CHAPTER 3

PLASMID DNA ANALYSIS OF *BACTEROIDES* SPECIES

INTRODUCTION

The presence of plasmids in bacteroides is well established. However, because of the pathogenic role of B. fragilis, work has been centred around an examination of the fragilis group strains (Guiney and Davis, 1975; Rotimi et al., 1981; Wallace et al., 1981; Welch et al., 1979; Young and Mayer, 1979). To our knowledge there is no published data on the incidence of plasmids in Bacteroides of the melaninogenicus/oralis and asaccharolytic groups. The aims of this study were firstly, the development of a plasmid isolation technique suitable for Bacteroides species and secondly; the determination of the incidence of plasmids in strains representative of the three Bacteroides groups.

(a) Development of a Method for the Isolation and Visualisation of Plasmids in Bacteroides species.

The ideal method was required to be fast and simple (as large numbers of strains were to be screened), producing accurate and reproducible results. Several methods were considered but as resistance to digestion by lysozyme has been reported for B. fragilis cells (Werner, 1981) methods involving lysozyme were initially avoided.

The Method of Kado and Lui (1981)

The method of Kado and Lui [(1981) Fig 3.1)] was first attempted. This method was developed for the isolation of plasmids from several aerobic and facultatively anaerobic species, including

3 ml O/N broth culture (OD 0.8 at 600 nm) — [100 ml O/N broth culture]*

↓

Pellet cells at 5,700 rpm, 4°C, 7 min — [2,500 g, 20 min room temperature]

↓

Resuspend in 1 ml buffer E (40 mM Tris-acetate, 2 mM sodium EDTA pH 7.9 with glacial acetic acid)

↓

Add 2 ml of lysing solution (3% SDS and 50 mM Tris pH 12.6) and mix by gentle agitation

↓

Heat 50 - 65°C, 20 min — [60°C, 5 min]

↓

Emulsify lysate with 2 volumes phenol-chloroform solution (1 : 1 v/v)

↓

Break emulsion 6,000 rpm, 15 min 4°C — [2,500 g, 30 min room temperature]

↓

25 ul upper aqueous phase + 10 ul tracking dye (0.25% bromocresol purple in 50% glycerol - 0.05 M Tris-acetate (pH 7.9)

↓

Horizontal gel (0.7% agarose) 12 µ/cm ~ 2 h — [70 V, 24 h]

↓

Stain with ethidium bromide (0.5 µg/ml) 30 min

↓

Transilluminator

Figure 3.1

The plasmid isolation technique of Kado and Lui (1981)

* Modifications made to the technique during this study

E. coli. Modifications (shown in parenthesis) were made to the published procedure.

The total volume of culture (3 ml) was found to be insufficient and was increased to 100 ml. Available Sorvall tubes were not phenol resistant, therefore, centrifugation was in a bench centrifuge with modifications in the gravitational force, time and temperature of the run.

The conditions for lysis covered a wide temperature range (50 - 65°C for 20 min). The effect of different temperatures and times on the isolation of DNA from bacteroides was investigated. In the absence of plasmid-containing strains the effect of variation on the chromosomal DNA of an oral strain of Bacteroides (isolated from the gums of a healthy individual) was investigated. The procedure was conducted at four temperatures (50, 55, 60 and 65°C) with four time intervals (5, 10, 15 and 20 min) at each temperature.

It was found that at 65°C the chromosomal DNA bands obtained were very faint or absent. At 60°C the chromosomal bands were clearly visible but faint after a period of 20 mins. At 55 and 50°C the chromosomal bands were present but decreased in intensity with increasing time. It was decided that lysis should be at 60°C for 5 min.

The plasmid profiles of 10 Bacteroides fragilis group strains (clinical isolates from the diagnostic laboratories of the Royal Infirmary of Edinburgh), designated T1 to T10 were investigated. Viscous lysates were obtained from some of the cultures, while with others, cell suspensions remained turbid and lysis appeared to be

minimal. Plasmids were not observed in any of the strains and DNA from cultures with viscous lysates appeared as streaks in the gel. It was decided that the method of Kado and Lui (1981) was unsuitable for the isolation of plasmids from bacteroides. However due to the simplicity of the technique and the lysozyme-free method of lysis it was decided that the closely related plasmid isolation technique of Plazinski et al. (1983) should be the next investigated.

The Method of Plazinski et al., (1983)

The method of Plazinski et al. (1983) was a modification of that of Kado and Lui (1981) (Fig 3.2). It included a washing step for the bacterial cells and utilised the lysing solution of Kado and Lui (1981) with modifications in the lysing procedure. The plasmid profiles of the 10 Bacteroides fragilis group strains designated T1 to T10 were investigated.

Plasmids were observed in three of the strains tested (Fig 3.3). However viscous lysates were produced by eight strains and as before this resulted in streaks of DNA on the gel. The effects of aerobic and anaerobic conditions on the method of preparation were investigated. Duplicate samples were prepared, one on the bench and the other in an anaerobic cabinet (Forma Workstation, Model 1028) but no difference was found in the plasmid profile or the quality of the plasmid preparations. It was concluded that future preparations could be done on the bench. However it was decided that the methods of Kado and Lui (1981) and Plazinski et al. (1983) though suitable

10^{10} cells from O/N broth culture

pellet cells at 5,7000 rpm 4°C, 7 min — [100 ml O/N broth culture]* [2,500 g 20 min, room temperature]

↓

Resuspend and wash in 0.5 ml TAE buffer [40 mM Tris, 5 mM sodium acetate, 1 mM ETDA disodium salt (pH 7.8)]

↓

Add 2 ml lysing solution (Kado and Lui, 1981) and mix by gentle agitation

65°C, 1 h then cool at room temperature, 5 min — [60°C, 5 min]

↓

Add, with shaking, 0.25 ml of 2 M Tris (pH 7.0) and 5 ml phenol-chloroform solution 1 : 1 v/v

↓

Centrifuge 7,500 rpm 15 min, 0°C — [3,989 g, 30 min room temperature]

↓

70-140 ul upper aqueous phase + tracking dye [50% glycerol, 50mM Tris acetate (pH 7.8) 0.125% bromophenol blue] — [3 ul upper aqueous phase + 10 ul tracking dye (Kado and Lui, 1981]

↓

Horizontal gel, 44 V for 1 h then 120 V, 10 - 12 h, 4°C — [0.7% agarose, 70 V for 24 hr]

↓

Stain with ethidium bromide (1 µg/ml), 60 min — [Stain in E + Br 0.5 µg/ml 30 min (Kado and Lui, 1981) and observe directly on a transilluminator]

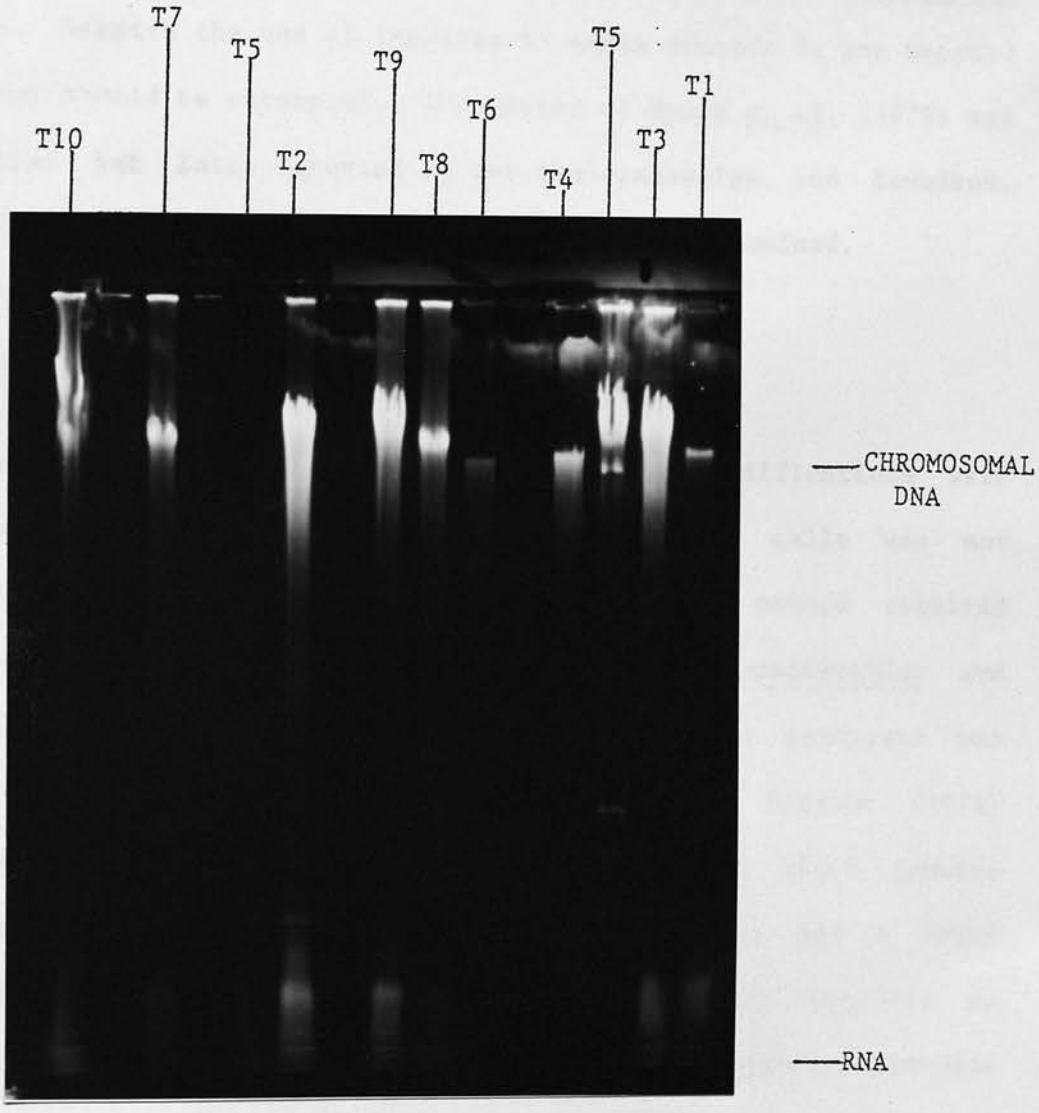
↓

Destain in TAE buffer containing 1 mM $MgSO_4$, 30 min

Figure 3.2

The plasmid isolation technique of Plazinski et al. (1983)

* Modifications to the technique made during this study



gure 3.3 Agarose gel (0.7%) showing plasmids isolated from Bacteroides by the modified method of Plazinski et al. (1983). Electrophoresis was at 70 V for 24 h, at 4°C.

for Gram-negative aerobes and facultative anaerobes, were not very suitable for Bacteroides.

The failure of these methods prompted an examination of methods developed specifically for isolation of plasmids for Bacteroides species. Despite the use of lysozyme in these methods it was decided that they should be attempted. The method of Myers et al. (1976) was considered but later rejected as too time-consuming and involved. The method of Tinnell and Macrina (1976) was then examined.

The Method of Tinnell and Macrina (1976)

This method is outlined in Fig 3.4. Basic modifications were considered necessary. Thymidine labelling of the cells was not required so the first two steps were omitted. The method required was one for simple screening of crude lysates of Bacteroides and purification on caesium chloride - ethidium bromide gradients was therefore inappropriate. The method of Tinnell and Macrina (1976) was combined with that of Guerry et al. (1973), in which lysates incubated overnight were cleared of cell debris and a large percentage of chromosomal DNA and plasmids subsequently separated on sucrose gradients. The sucrose gradients were avoided by treatment of the samples by the method of Kado and Lui (1981) after clearing of the lysates.

Results and Discussion

The modified method of Tinnell and Macrina (1976) used initially for the isolation of plasmids from Bacteroides is outlined in

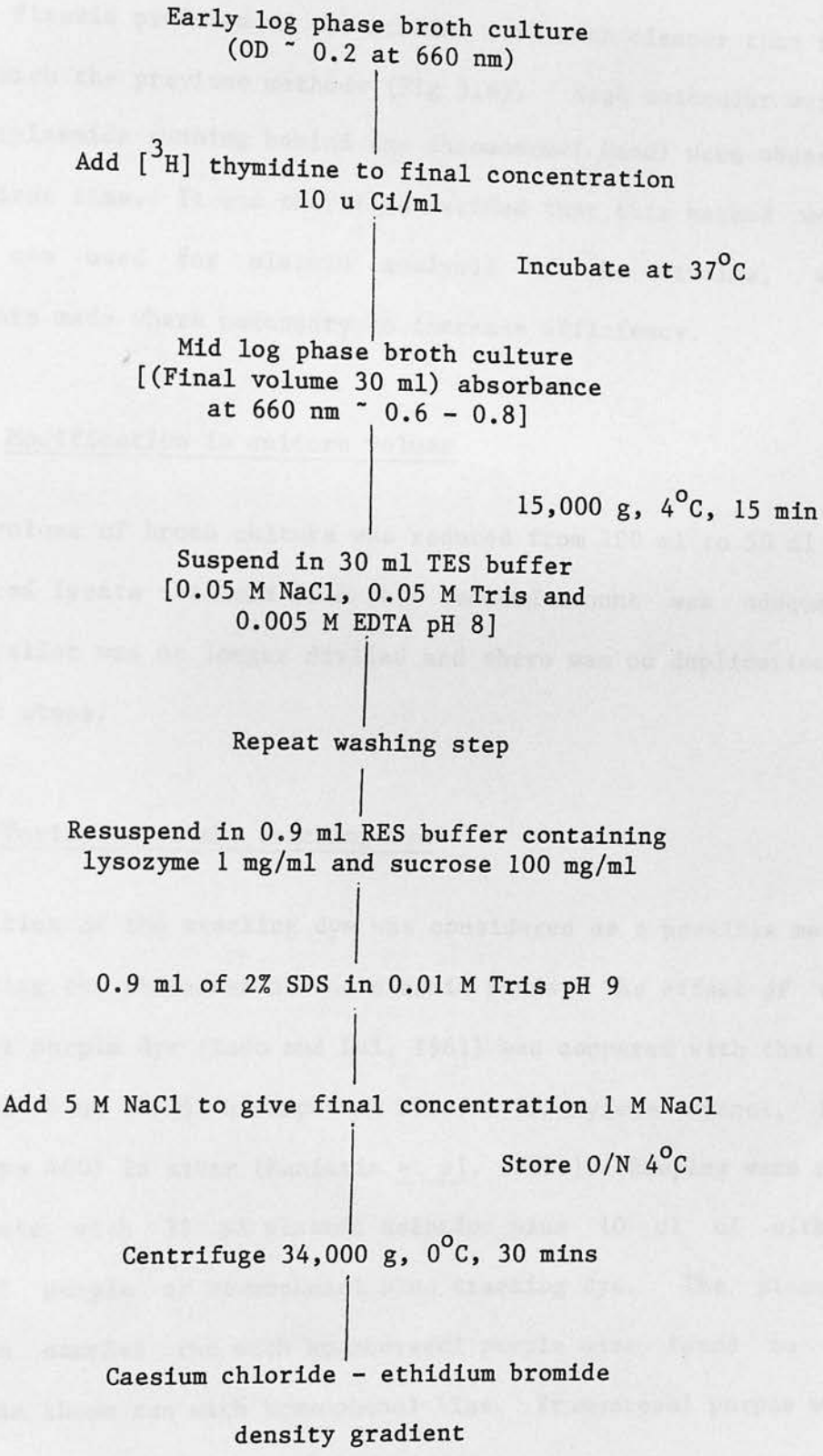


Figure 3.4

The plasmid isolation technique of Tinnell and Macrina (1976)

Fig 3.5. Plasmid profiles of the strains were much cleaner than those obtained with the previous methods (Fig 3.6). High molecular weight plasmids (plasmids running behind the chromosomal band) were observed for the first time. It was therefore decided that this method would be the one used for plasmid analysis of the strains, with improvements made where necessary to increase efficiency.

(i) Modification in culture volume

The volume of broth culture was reduced from 100 ml to 50 ml as the cleared lysate obtained from this reduced amount was adequate. The cell pellet was no longer divided and there was no duplication of subsequent steps.

(ii) Variation of the tracking dye

Variation of the tracking dye was considered as a possible means of increasing the sharpness of the plasmid bands. The effect of the bromocresol purple dye (Kado and Lui, 1981) was compared with that of bromophenol blue [0.25% bromophenol blue, 0.25% xylene cyanol, 15% (Ficoll type 400) in water (Maniatis et al. 1982)]. Samples were run in duplicate with 35 µl plasmid solution plus 10 µl of either bromocresol purple or bromophenol blue tracking dye. The plasmid bands from samples run with bromocresol purple were found to be sharper than those run with bromophenol blue. Bromocresol purple was therefore retained as the tracking dye.

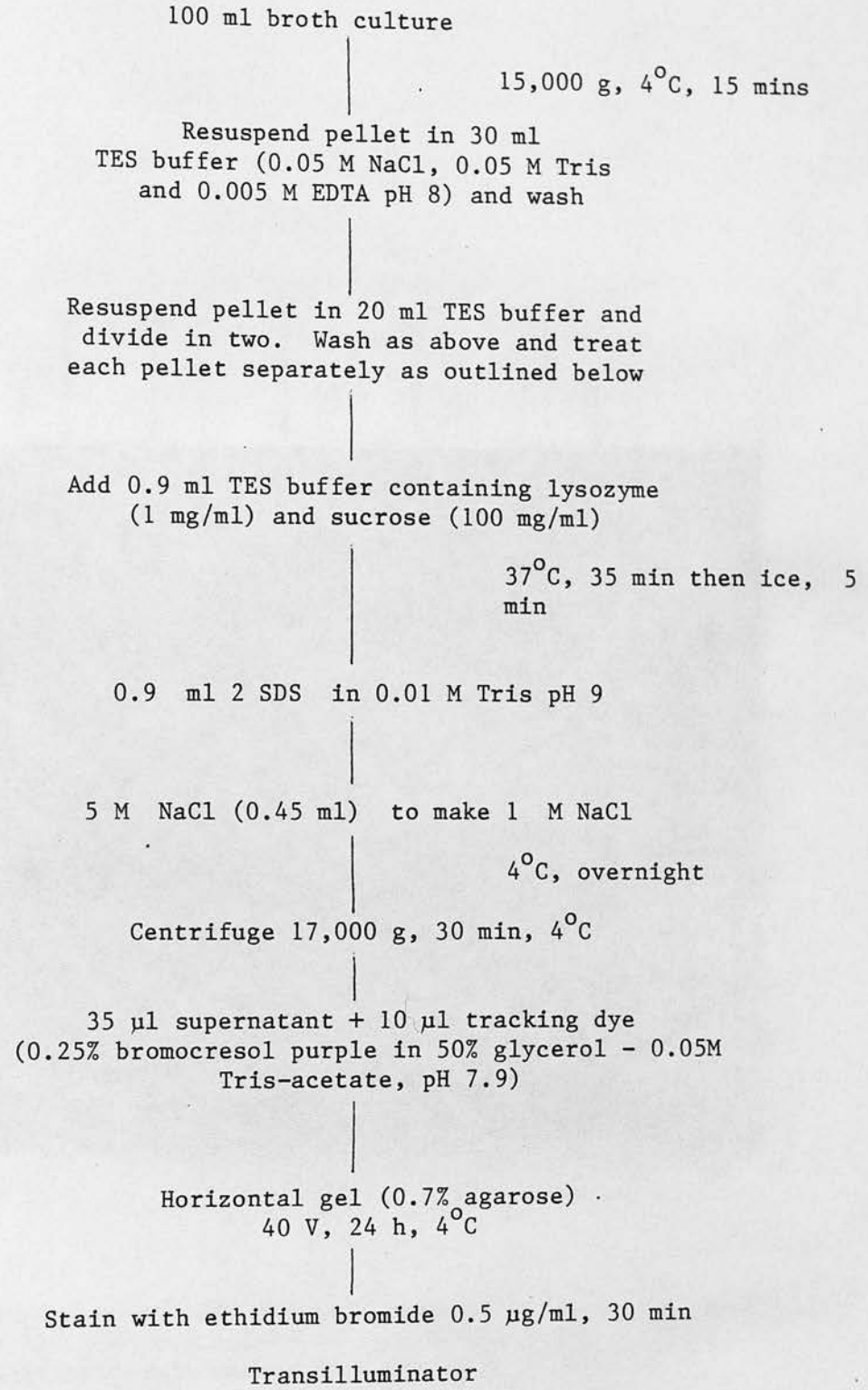


Figure 3.5

The modified method of Tinnell and Macrina (1976) for the isolation of plasmids from Bacteroides species.

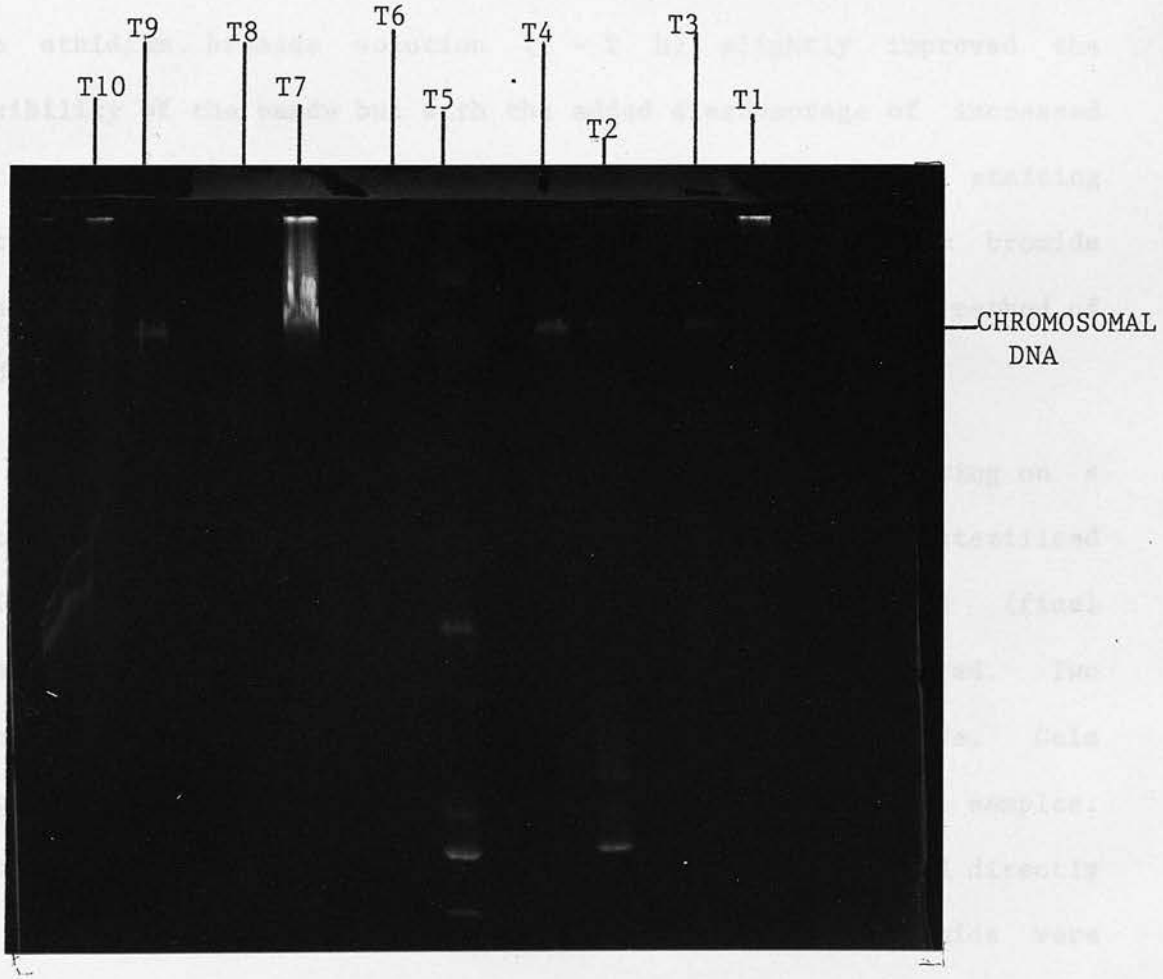


Figure 3.6 Agarose gel (0.7%) showing plasmids isolated from Bacteroides by the modified method of Tinnell and Macrina (1976). Electrophoresis was at 40 V for 24 h, at 4°C.

(iii) Modifications to the staining technique

The method of staining employed involved the incorporation of ethidium bromide into the DNA after separation in the agarose gel (Kado and Lui, 1981). However the bands were often faint after staining for the recommended time (30 min). Prolonged exposure to the ethidium bromide solution (1 - 2 h) slightly improved the visibility of the bands but with the added disadvantage of increased background haze. It was decided to investigate the staining technique of Maniatis et al. (1982) (in which the ethidium bromide was incorporated into the gel) and to compare this with the method of Kado and Lui (1981).

Agarose was dissolved in TES buffer ((1,000 ml) by heating on a water-bath) and cooled to approximately 55°C. A filter-sterilised solution of ethidium bromide (500 µg/ml) was added (final concentration ethidium bromide 1 µg/ml) and two gels poured. Two other gels were poured, as before, without ethidium bromide. Gels with and without ethidium bromide were loaded with duplicate samples. After electrophoresis gels with ethidium bromide were viewed directly on the transilluminator while those without ethidium bromide were stained as previously described.

Results

The results of the comparison of the staining techniques are shown in Figs 3.7 and 3.8. There was a marked difference in the effectiveness of both techniques. An increased number of DNA bands was visible when staining was by the method of Maniatis et al.

Figures 3.7 - 3.8

Agarose gels (0.6%) showing the results of alternative staining techniques for the visualization of plasmid DNA isolated from Bacteroides species by the modified method of Tinnell and Macrina (1976).

(i) Ethidium bromide added to gel (final concentration 1 ug/ml) prior to pouring and electrophoresis.

(ii) Gel stained for 30 min in ethidium bromide solution (concentration 0.5 ug/ml) after electrophoresis.

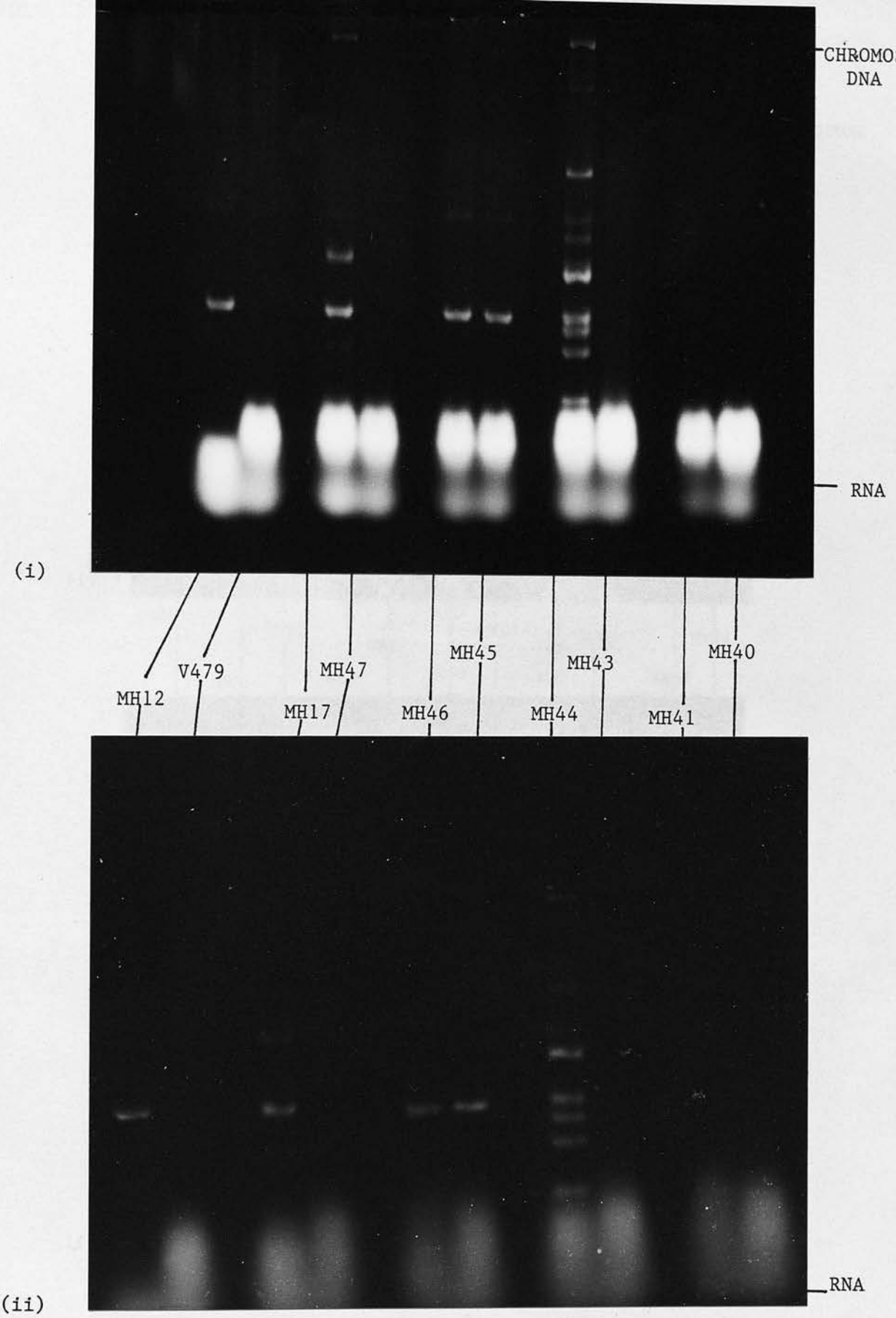

CHROMOSOMAL DNA
RNA
(i)
MH12
V479
MH17
MH47
MH46
MH45
MH44
MH43
MH41
MH40
RNA
(ii)

Figure 3.7

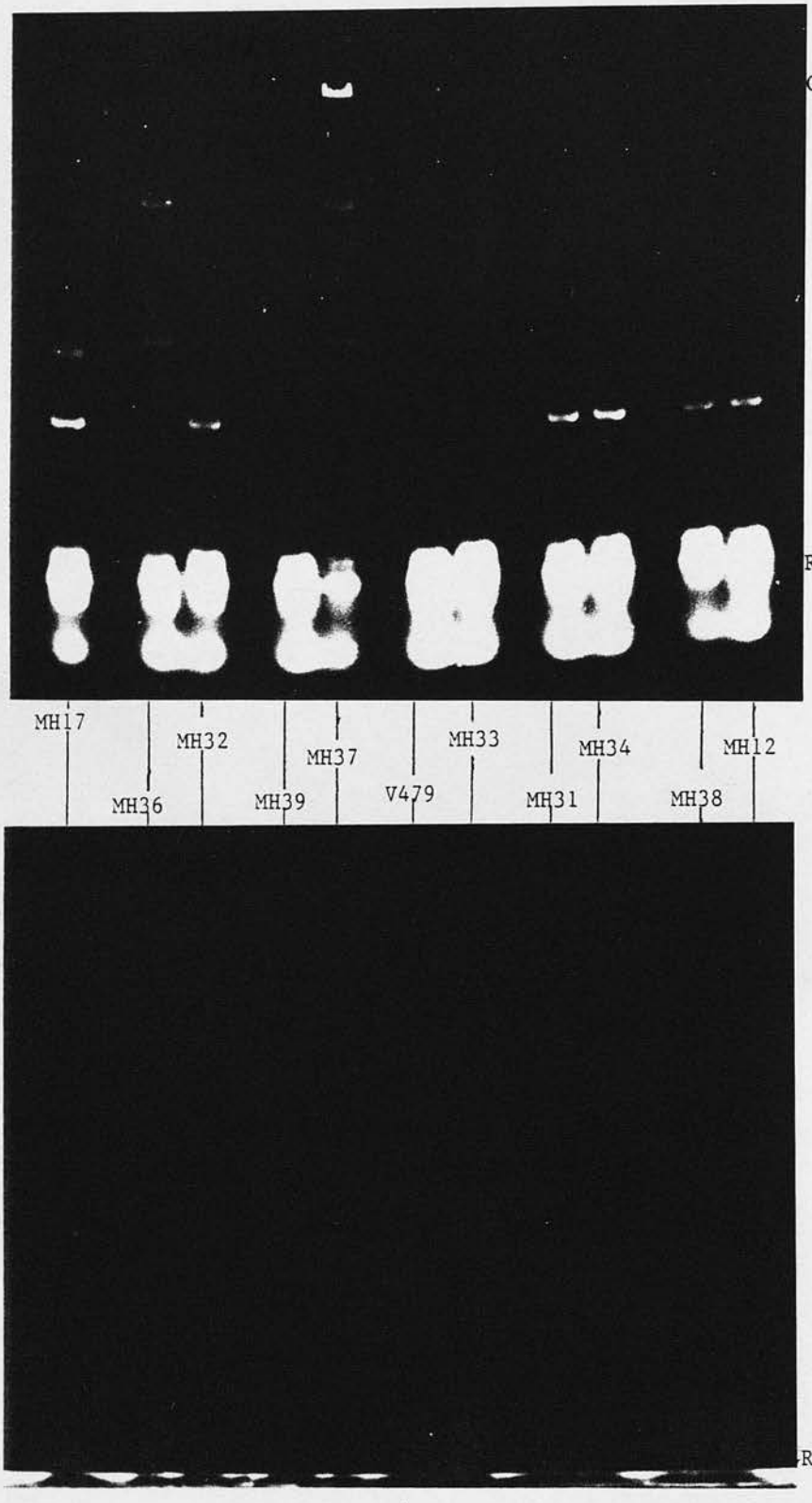

CHROMOSOMAL DNA
RNA
(i)
MH17
MH36
MH32
MH39
MH37
V479
MH33
MH31
MH34
MH38
MH12
RNA
(ii)

Figure 3.8

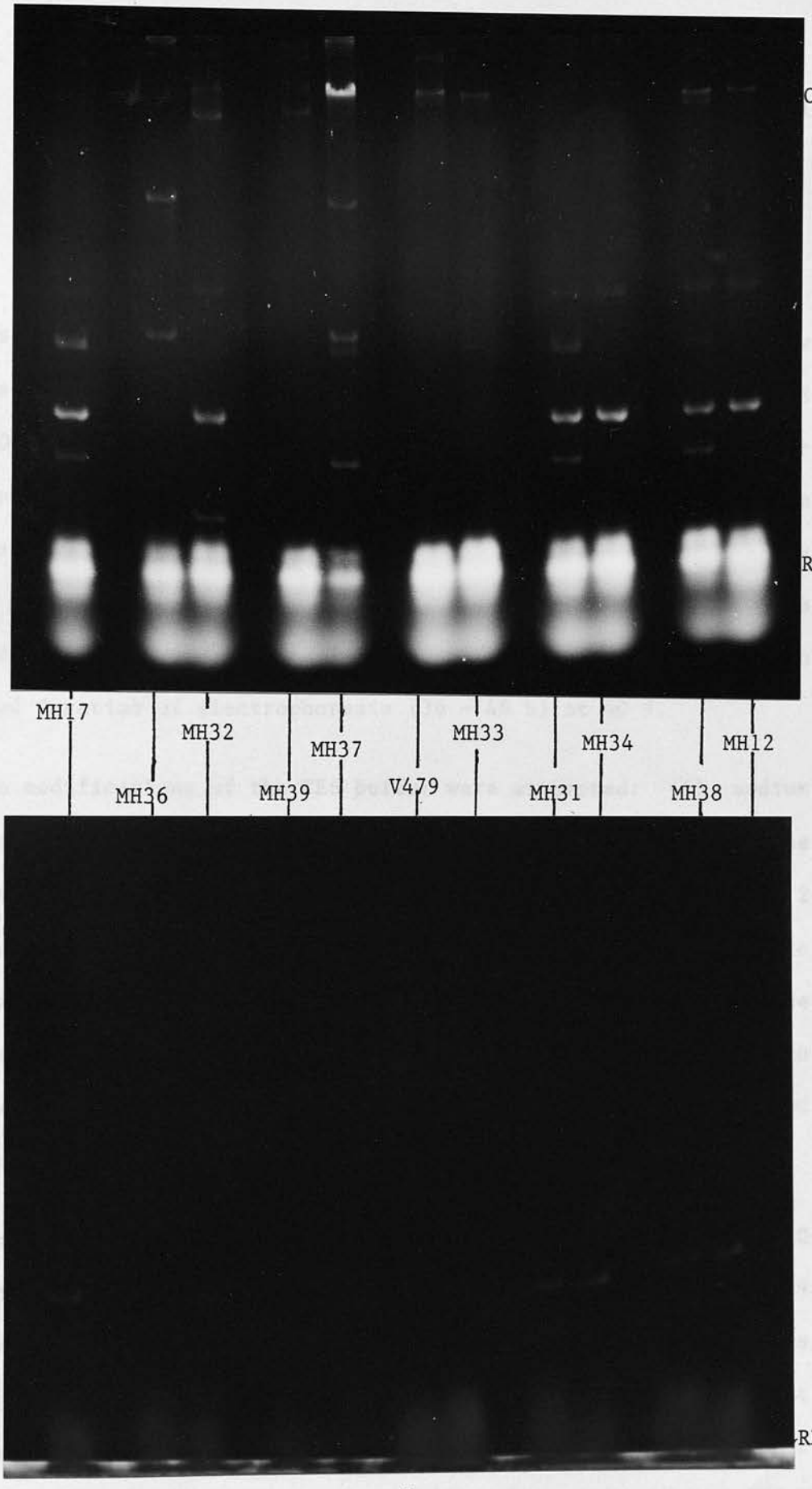

CHROMOSOMAL DNA
RNA
(i)
MH17
MH36
MH32
MH39
MH37
V479
MH33
MH31
MH34
MH38
MH12
RNA
(ii)

Figure 3.8

(1982). This method therefore replaced the method of Kado and Lui (1981).

(iv) Modification of the electrophoresis buffer

The high ionic concentration of the TES buffer resulted in low voltages with very high currents. A current of 80 mA was obtained with 40 V and migration distances of plasmids were low after electrophoresis for 24 h. Increasing the voltage to 70 V resulted in currents in excess of 100 mA and disassociation of the buffer (during the 24 h run) with dissolution of the gel in the region of the electrodes. Disassociation of the buffer was also observed with increased duration of electrophoresis (36 - 48 h) at 40 V.

Two modifications of the TES buffer were attempted: (i) sodium chloride was omitted as it was thought to be necessary only for the precipitation of chromosomal DNA; (ii) the buffer was diluted 1 : 2 (with distilled water) to investigate the need for a high ionic concentration of the electrophoresis buffer. In both cases the voltage:current ratio decreased but the stability of the high molecular weight plasmids was affected (some bands were lost) and there was a decrease in the resolution of the bands.

Tris-acetate buffer [0.04M Tris-acetate, 0.002 M EDTA, pH8.0 (Maniatis et al. 1982)] and Tris-borate buffer [0.089 M Tris, 0.0089 M boric acid, 0.0025 M EDTA, pH 8.0 (Myers et al. 1976)] were investigated. Low currents were obtainable with high voltages but the plasmid bands were diffuse. Excellent results were however obtained with the Tris-borate buffer [(TBE) 0.89 M Tris-acid, 0.089 M

boric acid, 0.002 M EDTA, pH 8.0) of Maniatis et al. (1982). It combined high voltages and low currents and gave good resolution of the plasmid bands. The electrophoresis buffer was changed to TBE.

(v) Variation in Agarose Concentration

Variation in the agarose concentration of the gels was investigated in an attempt to increase the migration distance of the plasmids. Due to the low voltages obtainable with the TES buffer and the restrictions on running time, it was decided to reduce the agarose concentration of the gels from 0.7% to 0.5%. However the lower agarose concentration resulted in a decrease in the resolution of the bands. When 0.6% agarose was investigated the migration distance of the plasmids was found to have increased without a corresponding decrease in the sharpness of the bands.

After the change of buffer from TES to TBE, 1% gels were investigated to ensure that large plasmids were not being left in the wells (as suggested by artefacts in the wells seen under ultra-violet illumination). Large plasmids were not detected but the relative mobilities of the plasmids were decreased. The agarose concentration was therefore standardised at 0.6%.

Final Method for the Isolation of Plasmid DNA from Bacteroides Species

The final method for the isolation of plasmid DNA was a modification of the method of Tinnell and Macrina (1976) (Fig 3.9).

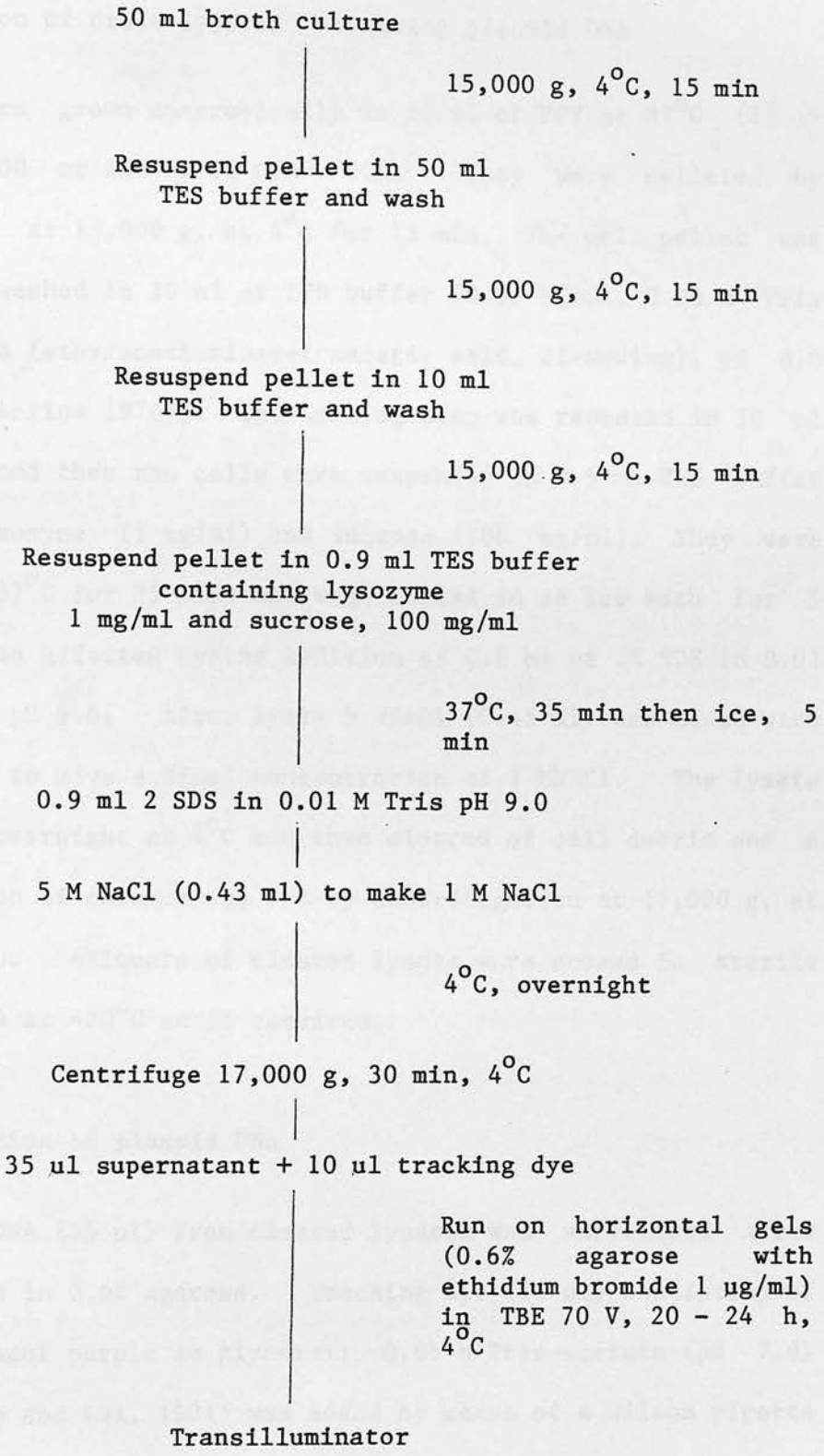


Figure 3.9

Final plasmid isolation technique - the modified method of Tinnell and Macrina (1976) for the isolation of plasmids from Bacteroides species.

(i) Preparation of crude lysates containing plasmid DNA

Cells were grown anaerobically in 50 ml of PPY at 37°C (18 - 48 h) to an OD at 600 nm of 0.6 - 0.8. They were pelleted by centrifugation at 15,000 g, at 4°C for 15 min. The cell pellet was suspended and washed in 30 ml of TES buffer [0.05 MCaCl, 0.05 M Tris and 0.5 mM EDTA (ethylenediaminetetraacetic acid, di-sodium), pH 8.0 (Tinnell and Macrina 1976)]. The washing step was repeated in 10 ml of TES buffer and then the cells were suspended in 0.9 ml TES buffer containing lysozyme (1 mg/ml) and sucrose (100 mg/ml). They were incubated at 37°C for 35 mins and then cooled in an ice bath for 5 min. Lysis was effected by the addition of 0.9 ml of 2% SDS in 0.01 M Tris acetate pH 9.0. After lysis 5 MNaCl (0.45 ml) was added with gentle mixing to give a final concentration of 1 MNaCl. The lysate was incubated overnight at 4°C and then cleared of cell debris and a large proportion of chromosomal DNA by centrifugation at 17,000 g, at 4°C for 30 min. Aliquots of cleared lysate were stored in sterile Eppendorf tubes at -20°C until required.

(ii) Visualisation of plasmid DNA

Plasmid DNA (35 µl) from cleared lysates was visualised after electrophoresis in 0.6% agarose. Tracking dye (10 µl) consisting of 0.25% bromocresol purple in glycerol: 0.05 M Tris-acetate (pH 7.9) (1:1 v/v) (Kado and Lui, 1981) was added by means of a Gilson pipette (with sterile tip) to the DNA sample in a sterile Eppendorf tube. The agarose was dissolved in 500 ml Tris-borate (TBE) buffer [(0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA pH 8.0) Maniatis et al, 1982]. Ethidium bromide solution [(500 µg/ml) filter

sterilised] was added to the cooled agarose (approximate temperature, 55°C) just before pouring of the gels, to give a final ethidium bromide concentration of 1 µg/ml.

All electrophoresis was performed at 4°C on horizontal gel apparatus (Fig 3.10). The dimensions of the gels were 19 x 20 cm. Gels were submerged in TBE buffer with a minimum of excess buffer covering the surface and 45 µl of sample (35 µl plasmid DNA solution + 10 ul tracking dye) was loaded into each well. Electrophoresis was at 100 V for 10 min (to run the samples into the gel) and then at 75 V for 20 - 24 h. The gels were then illuminated by a short wave UV transilluminator and photographed using Polaroid type 665 film or Kodak type 4147 film with a Wratten number 22 filter.

Storage of Buffers

All buffers and ethidium bromide stock solution were stored at 4°C. The tracking dye was stored at room temperature. Buffers were made as concentrated stock solutions (10 x working concentration). Immediately before use they were diluted and the pH adjusted as required.

(b) Incidence of Plasmid DNA in Bacteroides species

Materials and Methods

Bacterial strains

All strains in the Bacteroides culture collection were examined with the exception of MPRL 528, MPRL 610 and MPRL 628.

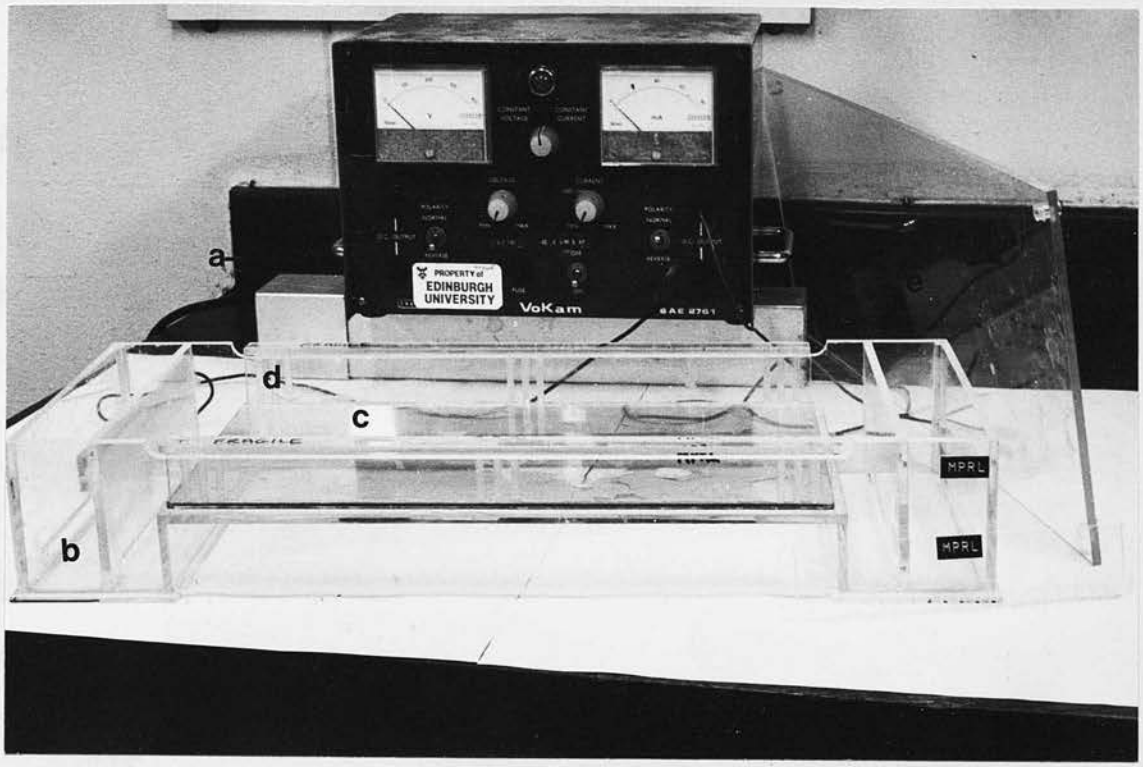
Figure 3.10

Horizontal gel apparatus used for agarose gel electrophoresis of plasmid DNA preparations,

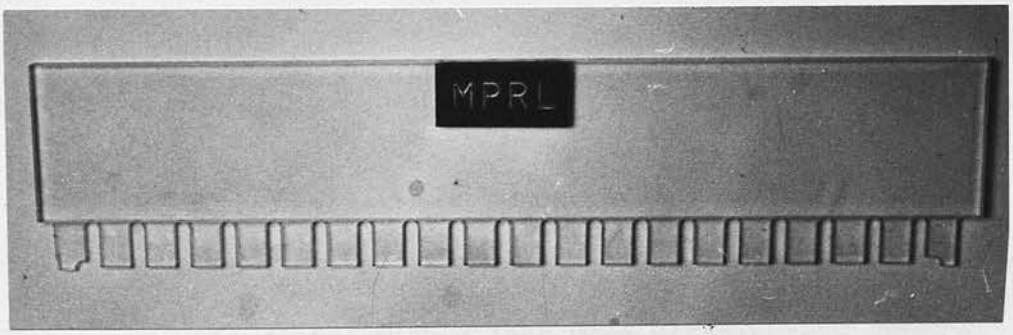
(i) Apparatus assembled for use showing:

(a) Power pack (Vokham SAE 2761)
(b) Gel tank
(c) Gel tray
(d) Slots for gel combs and spacers
(e) Gel tank cover

(ii) Gel comb designed to facilitate alternative method of well formation. Teeth at the extreme ends have extensions which raise the comb from the gel tray to allow formation of the bottom of the wells (modified from Maniatis et al. (1982) where teeth were the same length and coverslips were used to raise the comb from the gel tray).



(i)



(ii)

Extended teeth for well formation

Figure 3.10

Horizontal gel apparatus used for agarose gel electrophoresis of plasmid DNA preparations.

Plasmid Isolation and Visualisation

Plasmids were isolated and visualised by the modified method of Tinnell and Macrina (1976).

Preparation of Purified Plasmid DNA

Plasmid DNA in cleared cell lysates from 500-1000 ml broth cultures was purified by dye-buoyant density centrifugation by the modified method of Clewell and Helinski (1970). Centrifugation was performed on a Beckman model L2-65B ultracentrifuge in an SW-40 horizontal rotor at 150,000 g and 15°C for 95 hours or on a Sorvall OTD 65B ultracentrifuge in a fixed angle (Kontron 70.38) rotor at 210,000 g, and 15°C for 23 hrs.

Each polyallomer centrifuge tube contained 7.5 g caesium chloride, 6 ml of sample in TES buffer (4.5 ml sample + 1.5 ml TES) and 1.5 ml of a 1 mg/ml solution of ethidium bromide in TES. When smaller tubes were used, all amounts were proportionally reduced. The contents of the tubes were overlaid with light mineral oil.

After centrifugation the lower fluorescent band of these gradients was visualised with a long wave ultra-violet source. It was removed by side puncture with a needle followed by either aspiration into a syringe or by dripping into a clean tube. Ethidium bromide was removed by extraction with water saturated butanol. Equal volumes of butan-1-ol saturated with water were added with agitation (shaking or vigorous pipetting). The upper, pink, alcoholic phase was removed and the extraction repeated until this phase remained colourless after agitation. Plasmid DNA (contained in

the lower aqueous phase) was dialysed against TES buffer (2 litres) overnight with one change in buffer or for 4 - 8 h with two changes in buffer. It was then concentrated by precipitation with ethanol (modified method of Maniatis et al. 1982).

Two volumes of ethanol and one tenth of a volume of 2M sodium acetate were added to DNA solution in sterile Eppendorf tubes. The tubes were centrifuged at 4°C in an Eppendorf centrifuge (10,000 g). The supernatant was discarded and the tubes inverted on a layer of absorbent paper until dry. DNA from each tube was resuspended in 10 µl TES buffer. Purified plasmid DNA samples, for each strain, were pooled and stored separately at -20°C until required.

Determination of Molecular Mass of Plasmid DNA

The molecular weight mass of plasmid DNA was determined by the relative migration of plasmids in the test strains to plasmids of known molecular mass in the standard strains V479-1 and MH 44 (Fig 3.11). Migration distances were measured directly, with dividers and a ruler, from photographs of the gel. The distance measured was that from the bottom of the well to the leading edge of the plasmid bands.

Determination of Molecular Mass of Plasmids of Standard Strains

The standard strain B. fragilis V479 was known to have a single plasmid of molecular mass 27 MDa (Welch et al., 1979). The molecular mass of plasmids from B. fragilis MH44 were determined by agarose gel

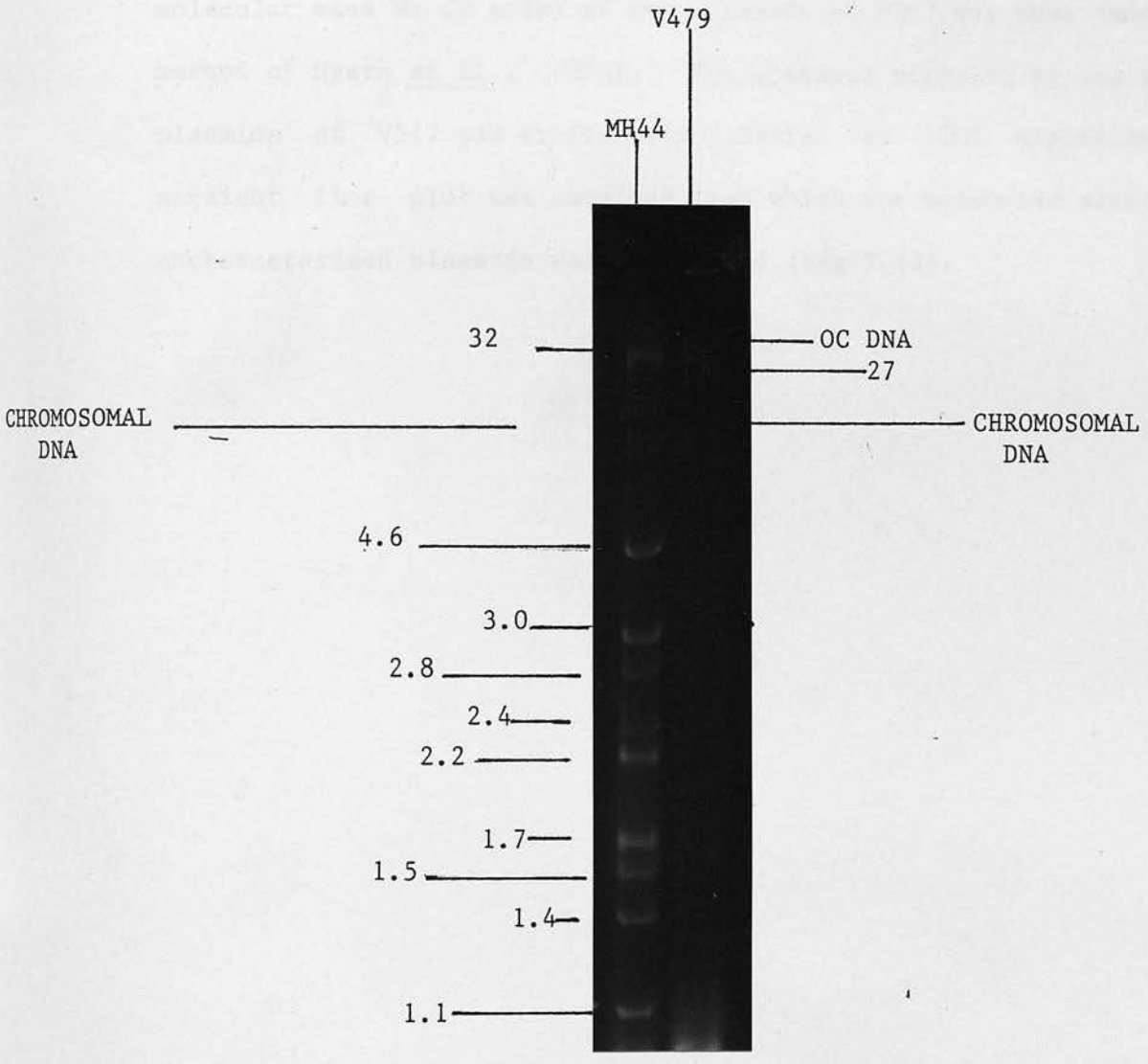


Figure 3.11

Agarose gel (0.6%) showing the plasmid profiles of the reference strains B. fragilis MH44 and B. fragilis V479. Numbers show molecular weight of plasmids in MDa.

electrophoresis of purified plasmid DNA using an E. coli strain V517 as a reference strain. V517 was known to contain eight plasmids ranging in molecular mass from 1.4 to 32 MDa (Macrina et al., 1978). A double logarithmic plot of relative migration (x axis) against the molecular mass Mr (y axis) of the plasmids of V517 was made (modified method of Myers et al., 1976). The distance migrated by one of the plasmids of V517 was arbitrarily selected as 100% migration. A straight line plot was obtained from which the molecular masses of uncharacterised plasmids was determined (Fig 3.12).

Typical logarithmic plot obtained for standard strain (B. fragilis MH44) for the calculation of molecular weights of uncharacterised plasmids.

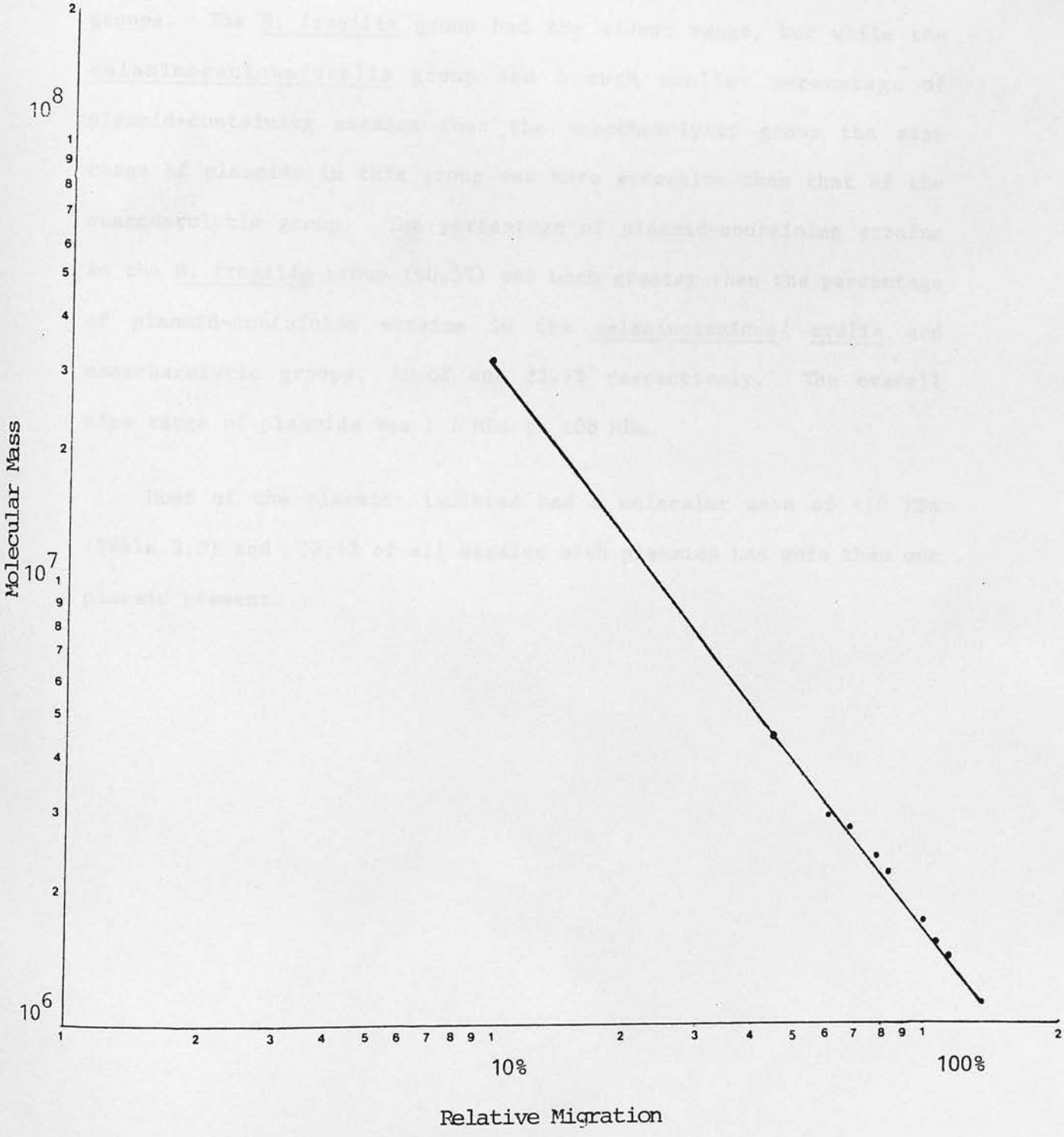


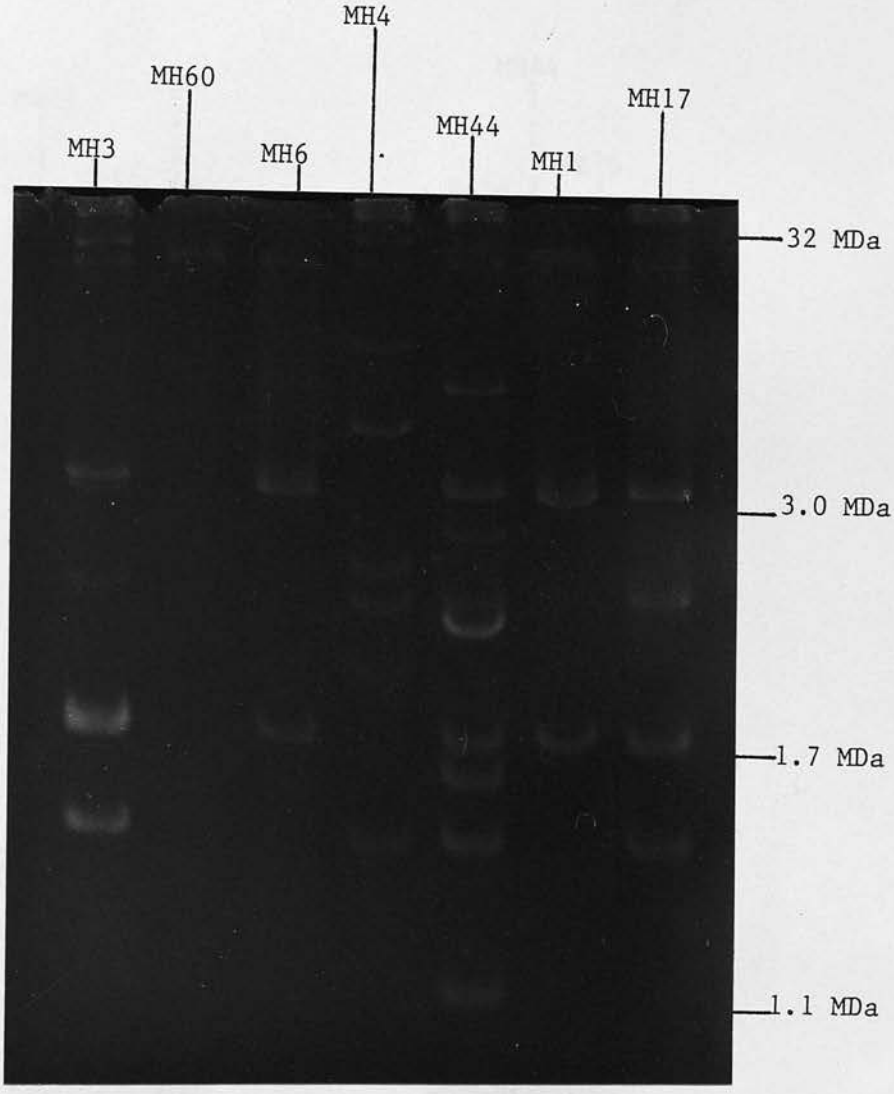
Figure 3.12

The plasmid profiles of strains in the Bacteroides culture collection are shown in Fig 3.13. The incidence of plasmids in each group is shown in Tables 3.1 and 3.2, and the incidence in each strain is shown in Appendix 2 but there was variation between the groups. The B. fragilis group had the widest range, but while the melaninogenicus/oralis group had a much smaller percentage of plasmid-containing strains than the asaccharolytic group the size range of plasmids in this group was more extensive than that of the asaccharolytic group. The percentage of plasmid-containing strains in the B. fragilis group (60.5%) was much greater than the percentage of plasmid-containing strains in the melaninogenicus/ oralis and asaccharolytic groups, 10.0% and 22.7% respectively. The overall size range of plasmids was 1.1 MDa to 108 MDa.

Most of the plasmids isolated had a molecular mass of <10 MDa (Table 3.2) and 32.4% of all strains with plasmids had more than one plasmid present.

Figure 3.13

Plasmid profiles of Bacteroides culture collection (pp 109-123)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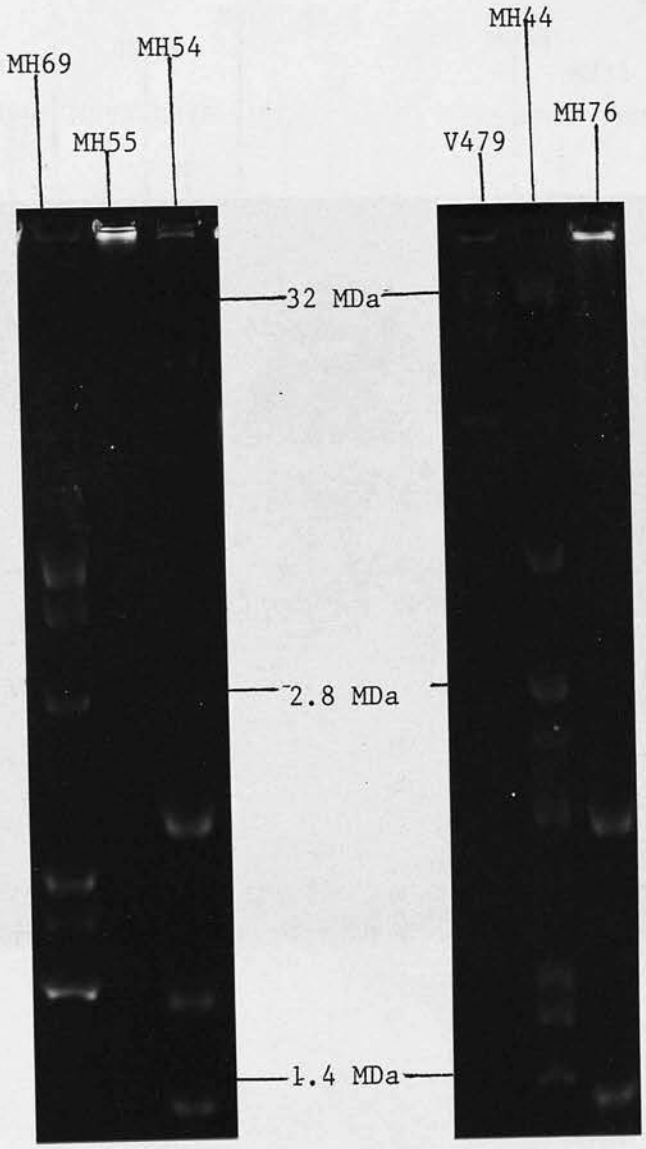
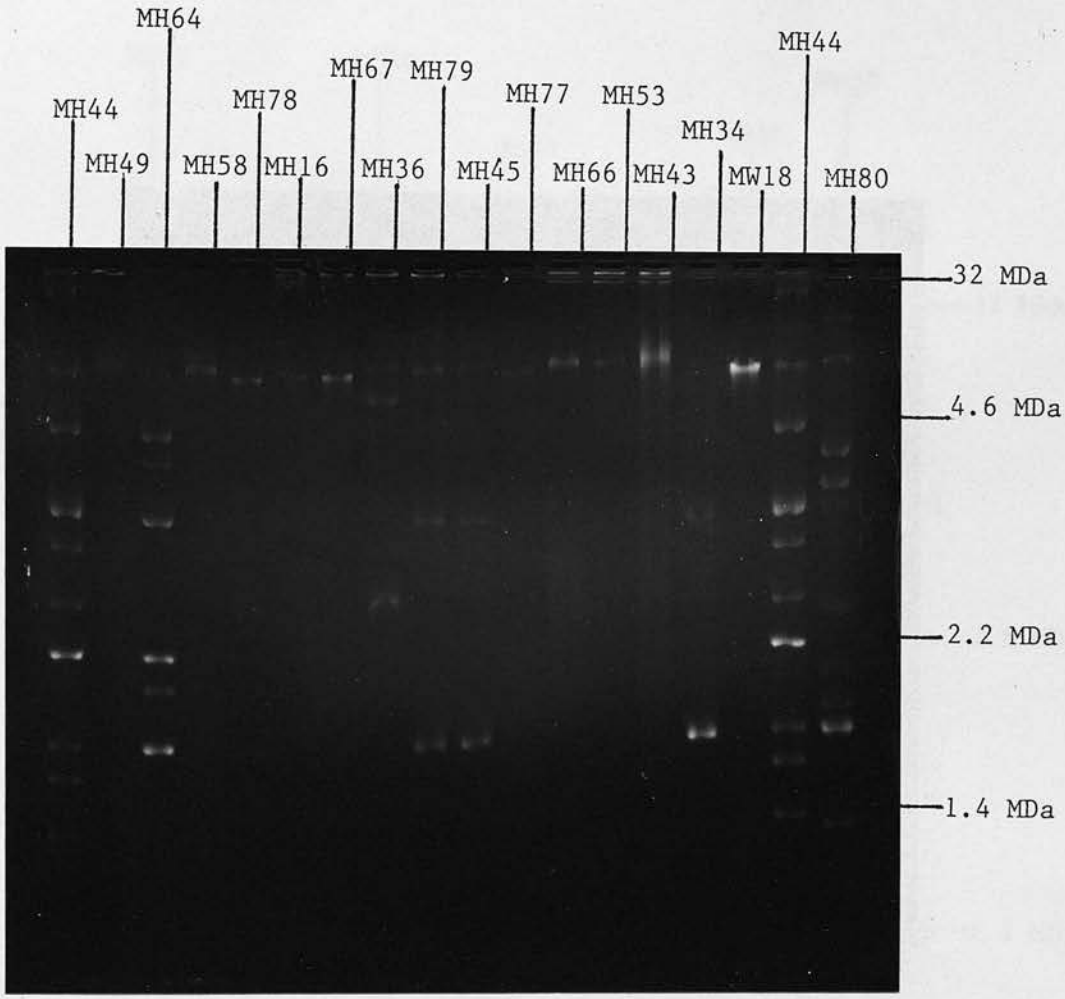
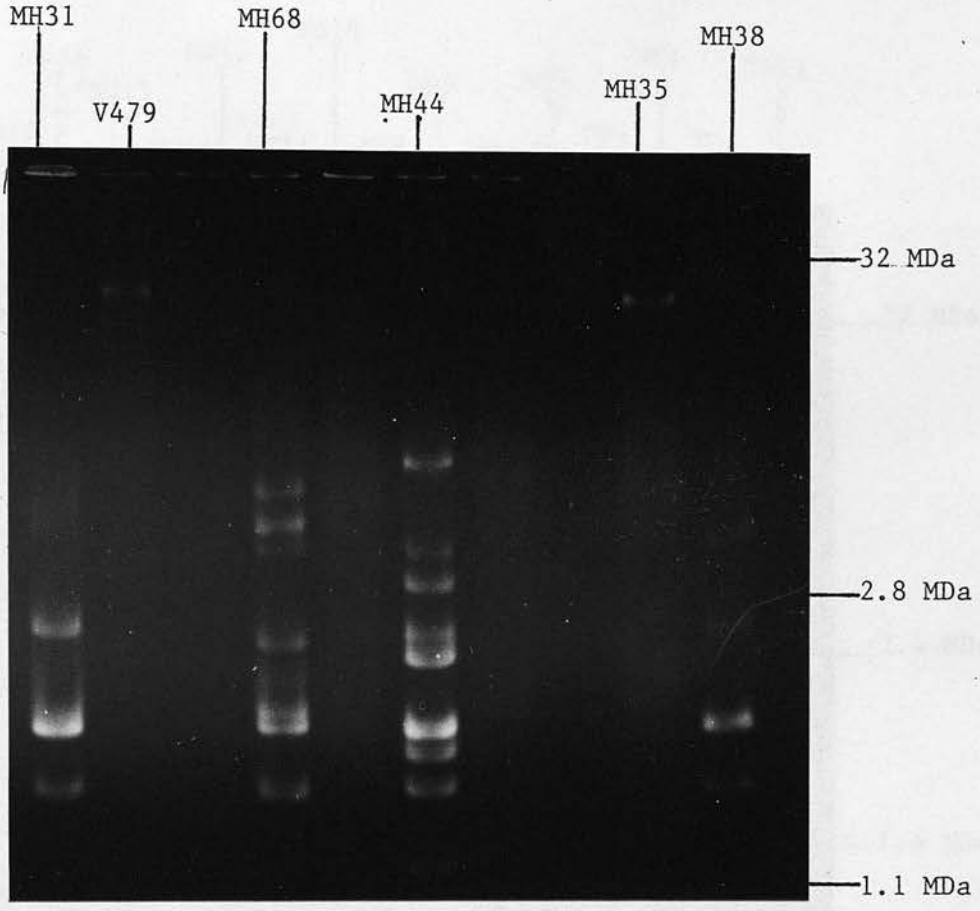
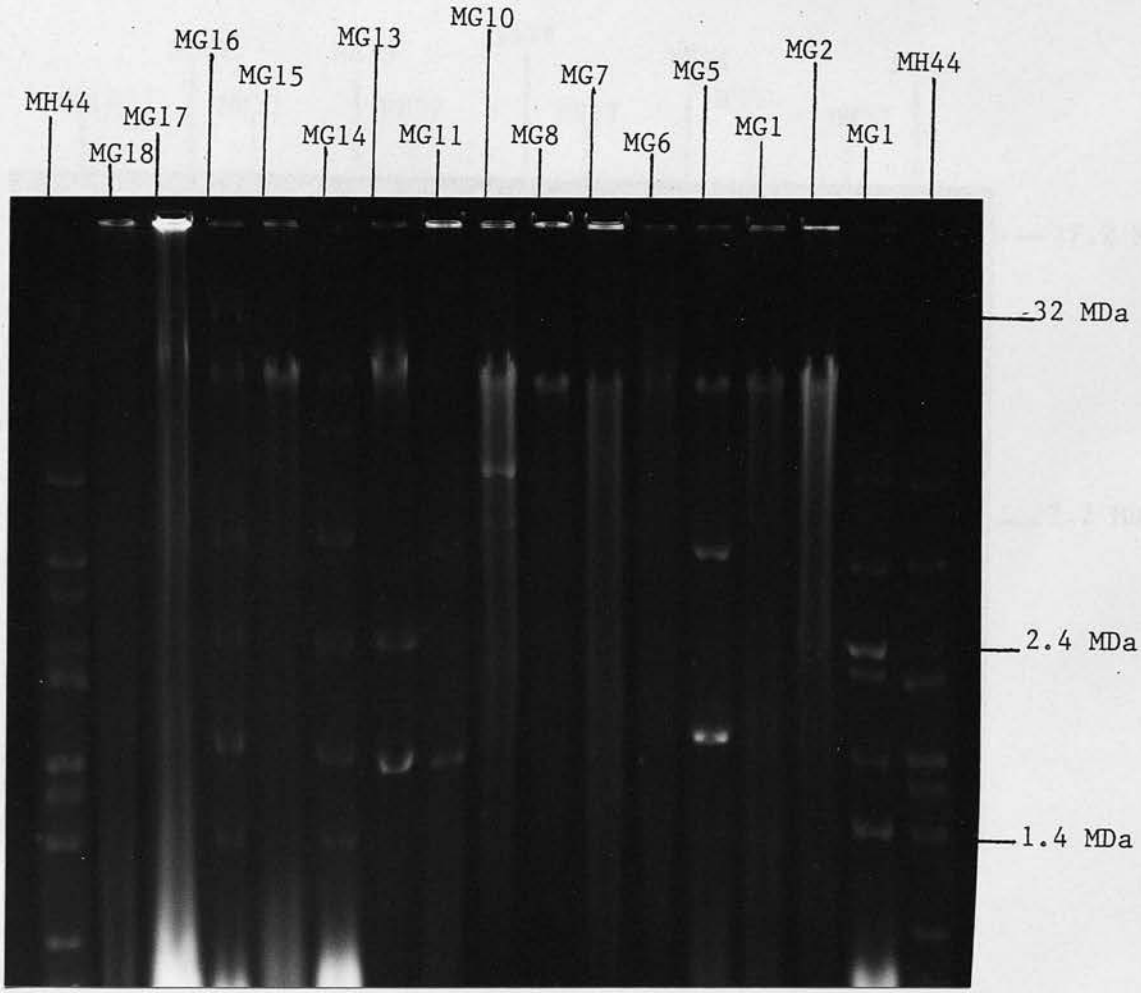
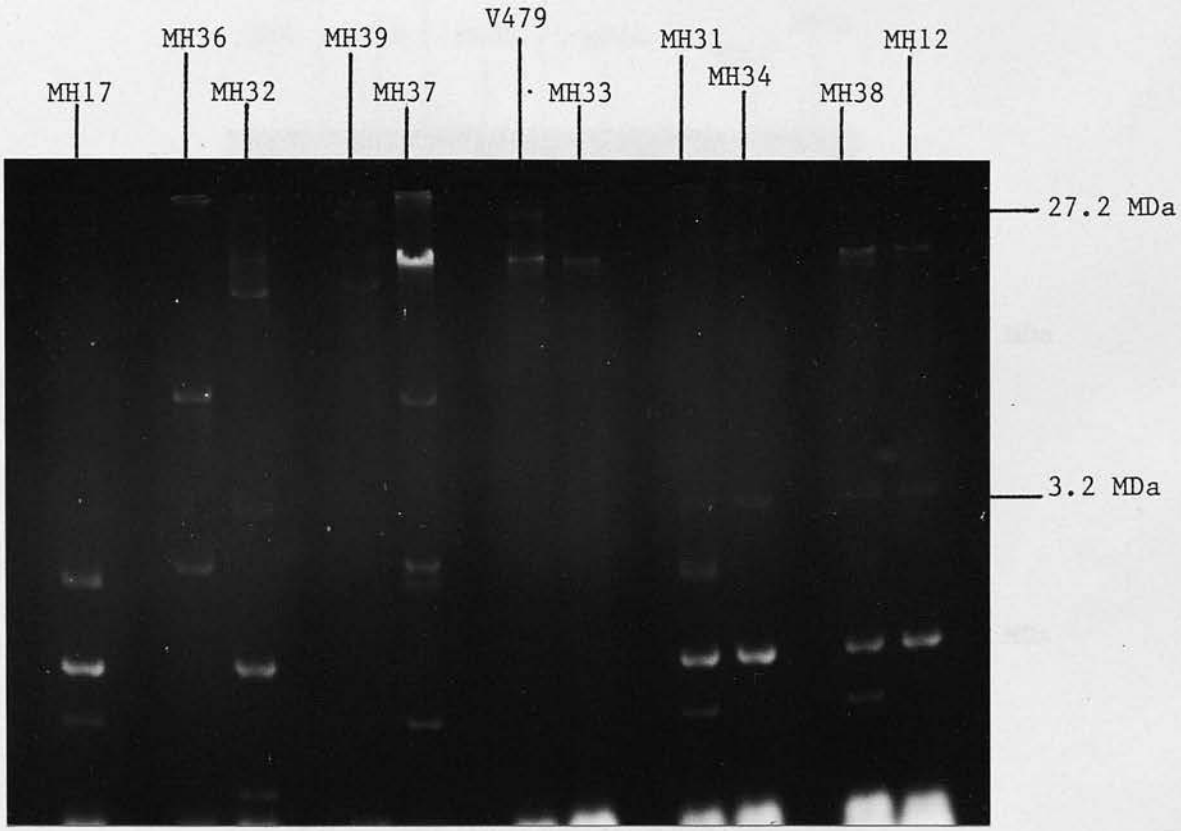
MH60
MH17
MH44
MH3
MH6
MH1
32 MDa
3.0 MDa
1.7 MDa
1.1 MDa

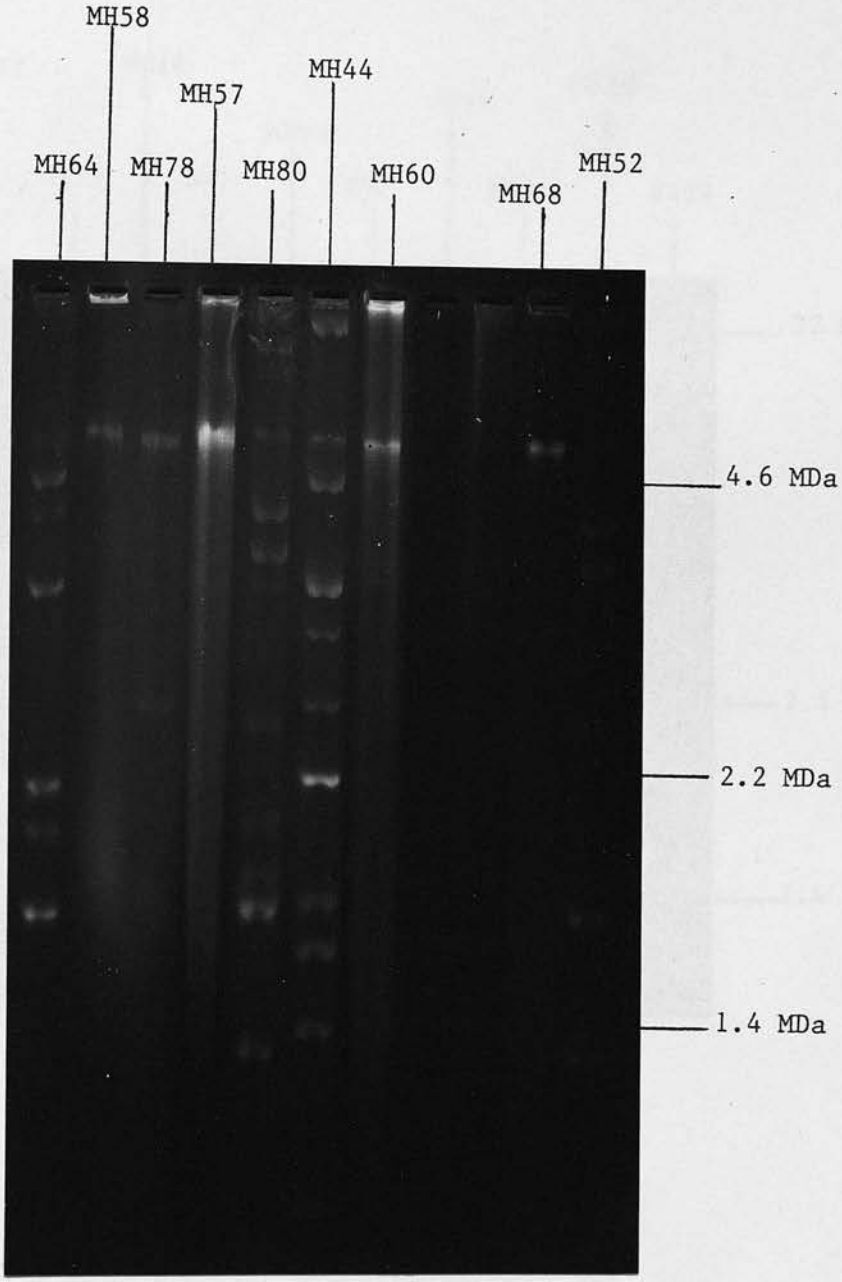

MH69
MH55
MH54
MH44
V479
MH76
32 MDa
2.8 MDa
1.4 MDa

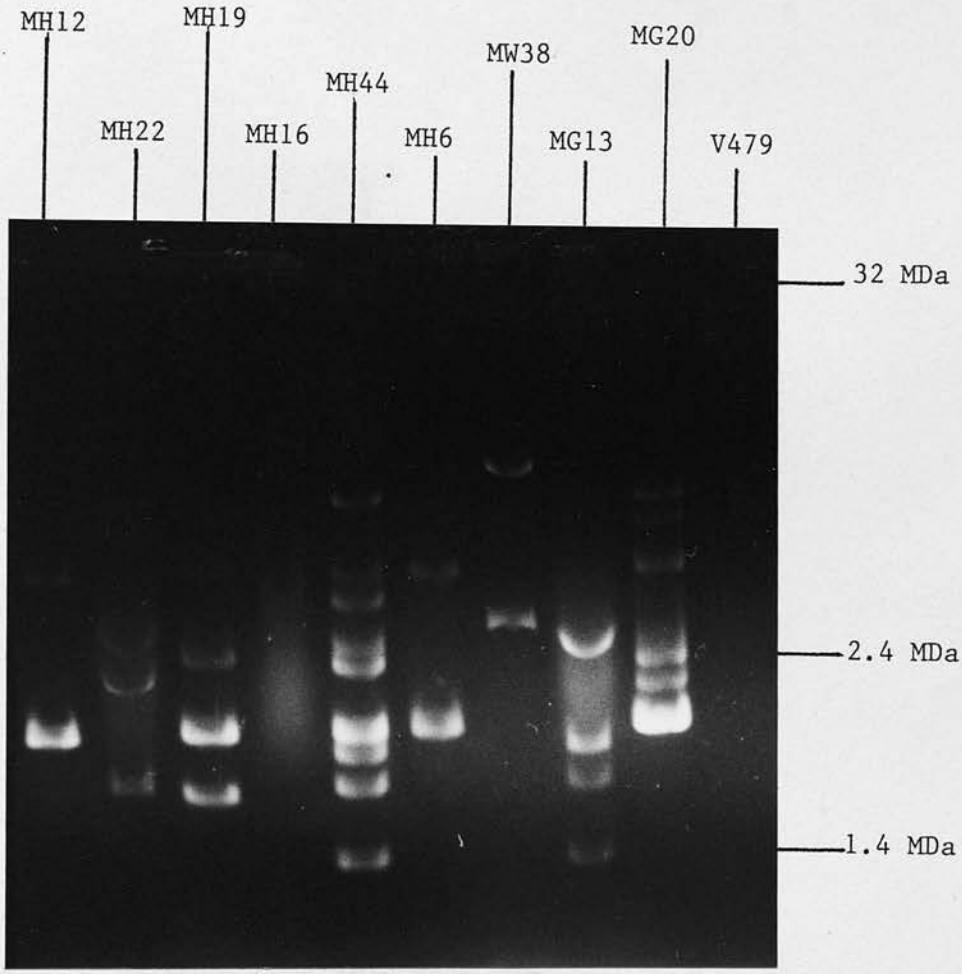

MH44
MH49
MH64
MH58
MH78
MH16
MH67
MH36
MH79
MH45
MH77
MH66
MH53
MH43
MH34
MW18
MH44
MH80
32 MDa
4.6 MDa
2.2 MDa
1.4 MDa

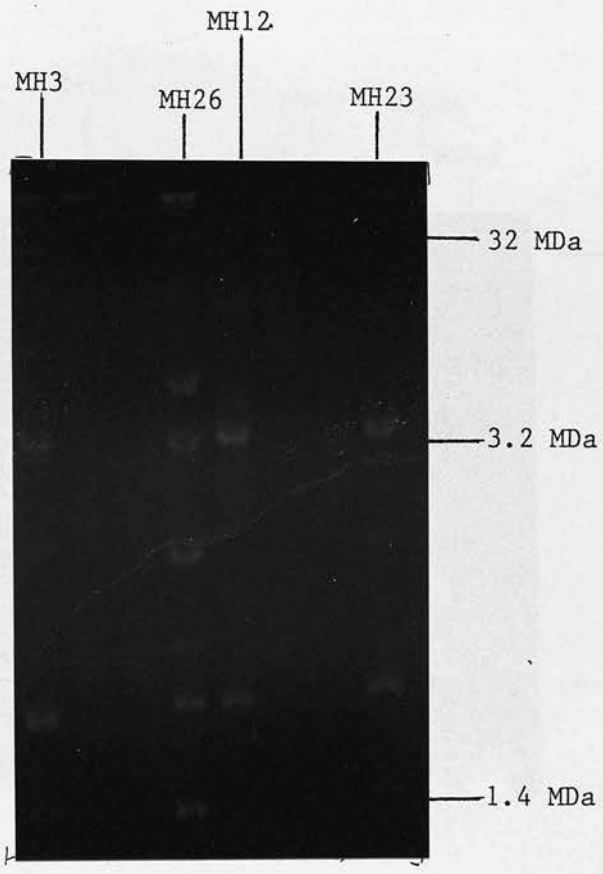

MH31
V479
MH68
MH44
MH35
MH38
32 MDa
2.8 MDa
1.1 MDa

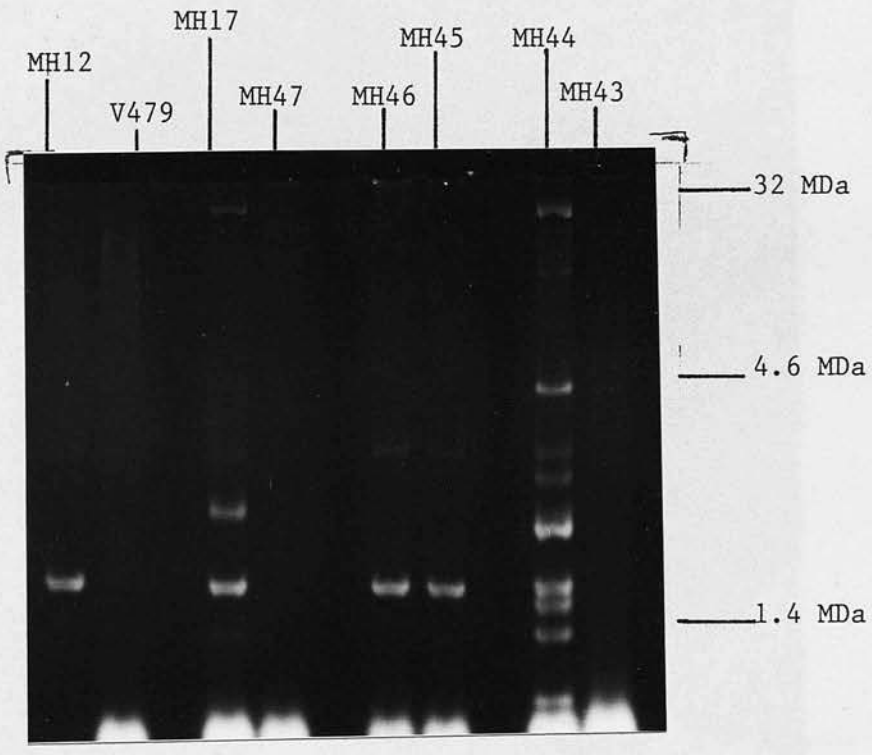

MH44
MG18
MG17
MG16
MG15
MG14
MG13
MG11
MG10
MG8
MG7
MG6
MG5
MG1
MG2
MG1
MH44
32 MDa
2.4 MDa
1.4 MDa

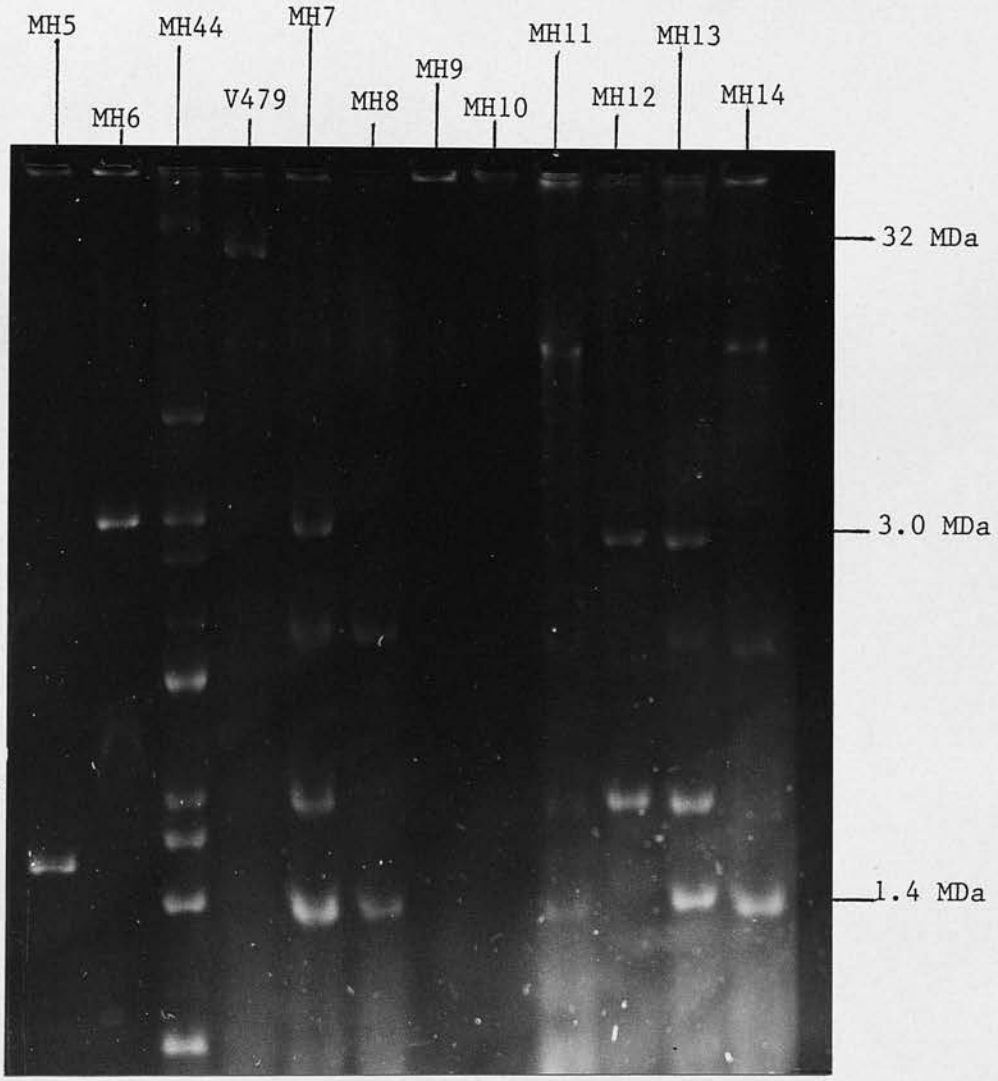

MH17
MH36
MH32
MH39
MH37
V479
MH33
MH31
MH34
MH38
MH12
27.2 MDa
3.2 MDa

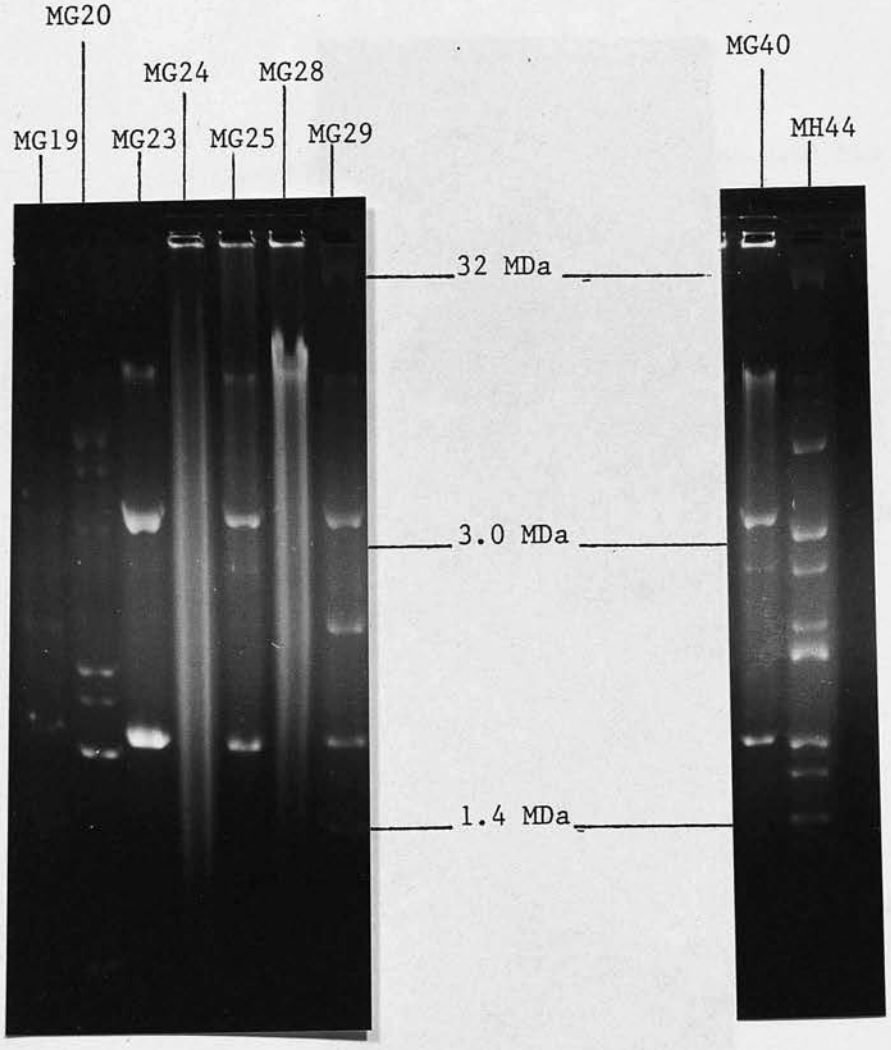

MH58
MH44
MH57
MH64
MH78
MH80
MH60
MH68
MH52
4.6 MDa
2.2 MDa
1.4 MDa

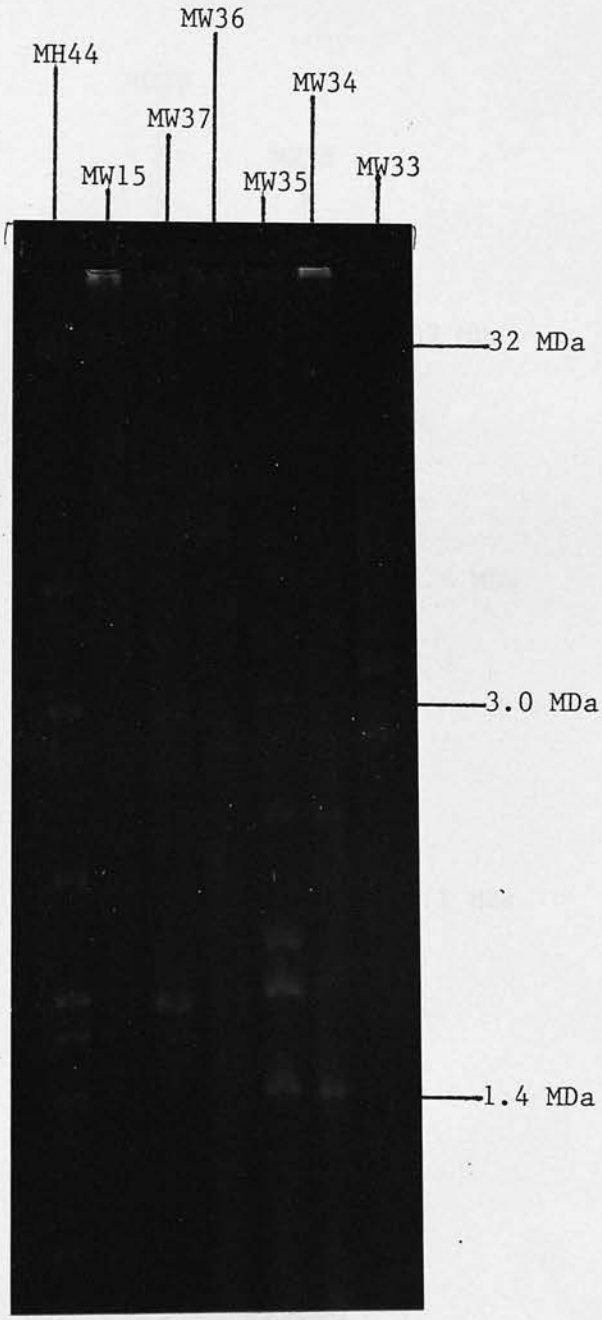

MH12
MH22
MH19
MH16
MH44
MH6
MW38
MG13
MG20
V479
32 MDa
2.4 MDa
1.4 MDa


MH12
MH3
MH26
MH23
32 MDa
3.2 MDa
1.4 MDa

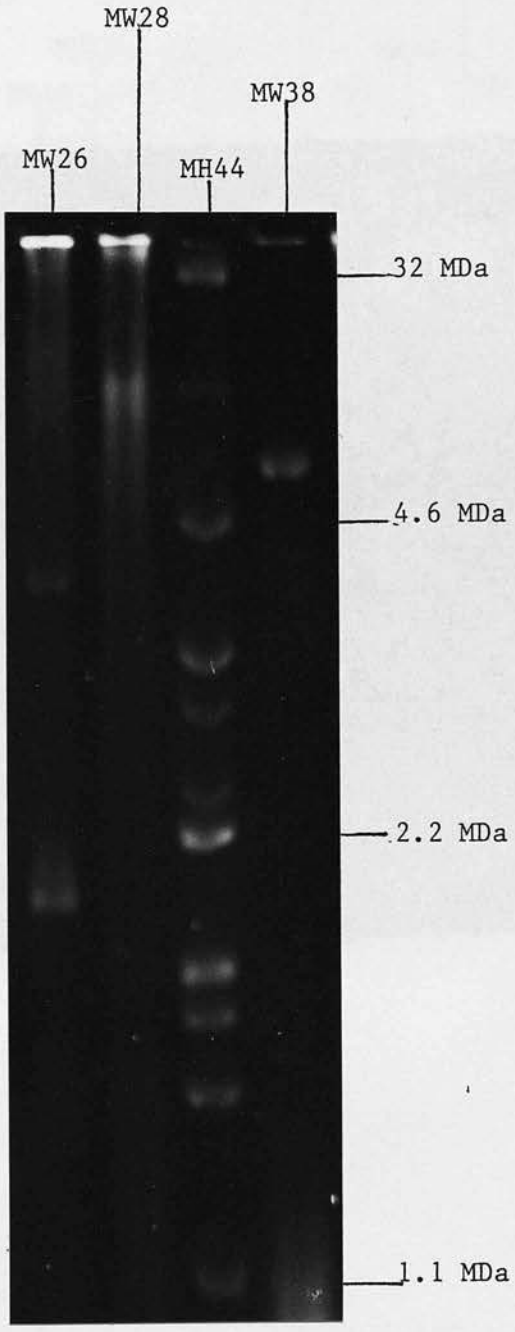

MH12
V479
MH17
MH47
MH46
MH45
MH44
MH43
32 MDa
4.6 MDa
1.4 MDa


MH5
MH6
MH44
V479
MH7
MH8
MH9
MH10
MH11
MH12
MH13
MH14
32 MDa
3.0 MDa
1.4 MDa


MG20
MG24
MG28
MG19
MG23
MG25
MG29
MG40
MH44
32 MDa
3.0 MDa
1.4 MDa



Plasmid profile of Bacteroides species


MW28
MW38
MW26
MH44
32 MDa
4.6 MDa
2.2 MDa
1.1 MDa

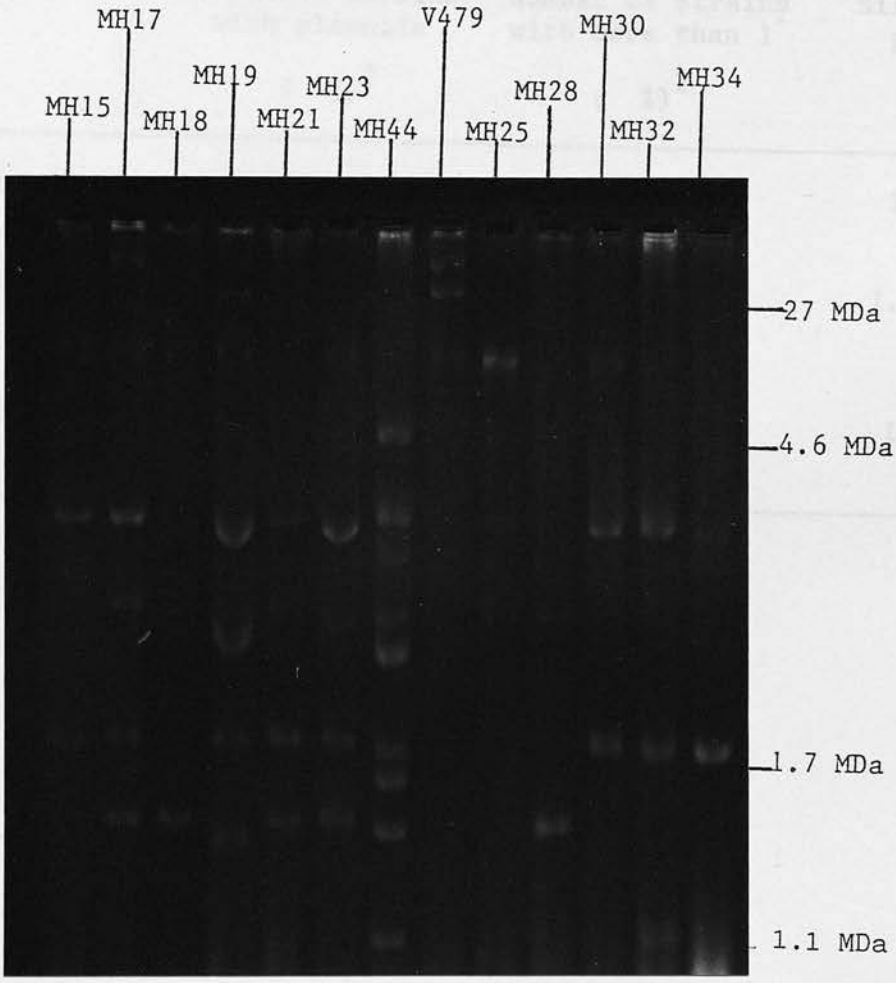

MH15
MH17
MH18
MH19
MH21
MH23
MH44
V479
MH25
MH28
MH30
MH32
MH34
27 MDa
4.6 MDa
1.7 MDa
1.1 MDa

TABLE 3.1 The Incidence of Plasmids in *Bacteroides* Species

Bacteroides group (n)[1]	Number of strains with plasmids (%)[2]	Number of strains with more than 1 (%)[2]	Size range of plasmids
fragilis (86)	52 (60.5)	42 (48.8)	1.1 - 108
melaninogenicus/ oralis (40)	4 (10)	1 (2.5)	1.75 - 32
asaccharolytic (22)	5 (22.7)	5 (22.7)	1.7 - 5.7

1 Number of strains in each group
2 Percentage of strains

TABLE 3.2 The Size Distribution of Plasmids in *Bacteroides* Species

Bacteroides group	Percentage of strains with plasmids of molecular mass (MDa)		
	<10	10 - 20	>20
fragilis (86)	58.1	5.8	13.9
melaninogenicus/ oralis (40)	7.5	-	2.5
asaccharolytic (22)	22.7	-	-

DISCUSSION

The determination of plasmid distribution in the genus Bacteroides was a prerequisite for the identification of plasmid-encoded virulence factors. The use of agarose gel electrophoresis for the detection and characterisation of bacterial extrachromosomal DNA (plasmids) has been well established. The results of this study emphasise the advantages of a technique developed specifically for the genus of the strains under investigation. Best results were obtained after modification of a method developed for Bacteroides SPP. The methods of Kado and Lui (1981) and Plazinski et al. (1983), though shown to give excellent results with E. coli and agrobacteria (S. Amyes and W. McLaughlin, personal communication), were found to be unsuitable for the isolation of plasmids from Bacteroides. Despite the report of Werner (1981), lysozyme was found to be an effective agent in the promotion of lysis in Bacteroides.

One of the limitations of the agarose gel electrophoresis method of detection of plasmid DNA is that it does not permit discrimination between open circular (OC) and covalently closed circular (CCC) DNA, (Myers et al., 1976). Macrina et al. (1978) reported the accumulation of small amounts of open circular molecules after prolonged storage at $4^{o}C$ or following repeated freezing and thawing of the samples. During this study samples were stored at $-20^{o}C$. Repeated freezing and thawing was avoided as it was found to destroy large plasmids. With the increased sensitivity of the staining technique OC molecules were recognised as very faint bands which were inconsistently present in uncharacterised strains or as additional

bands (usually faint) in DNA samples from strains of known plasmid content.

The molecular mass of plasmid DNA can be determined by its migration relative to plasmid DNA of known molecular mass (Myers et al., 1976). The multiple plasmid containing strain, E. coli V517 was reported to be a convenient single source of plasmid DNA of varying molecular sizes (Macrina et al., 1978). However the amount of plasmid DNA may vary between samples. The developed plasmid isolation technique was found to be more efficient at isolating plasmids from Bacteroides than from E. coli. The eight plasmids of V517 could not be visualised consistently after isolation procedures. It was therefore decided that the plasmids of a multiple plasmid-containing strain of Bacteroides, MH44, should be calibrated with reference to V517 and used with V479 as the standard strains.

Some of the results of this study confirm those of other workers. Most of the plasmid-containing strains had multiple plasmid populations (Riley and Mee, 1984; Young and Mayer, 1979) and most common plasmid sizes (10 MDa) were in keeping with published results (Riley and Mee, 1984; Wallace et al., 1981; Young and Mayer, 1979). However the overall size range of plasmids, 1.1 to 108 MDa, was greater that that reported in the literature. Plasmids with molecular mass of greater than 60 MDa have not previously been reported. The percentage of Bacteroides isolates with plasmids was greater than that reported in the literature. This may have been due to the increased sensitivity of the staining technique. Bands which would otherwise have remained undetected were visualised when ethidium bromide was incorporated into the gel as opposed to staining

after electrophoresis. The discrepancy could have resulted from the detection of low copy number plasmids which would by other staining methods produce very faint or undetectable bands.

The plasmid incidence of the melaninogenicus/oralis and asaccharolytic strains was markedly less than that of the B. Fragilis group strains. As the latter group are more often associated with infection this may indicate an as yet unestablished role of plasmids in the virulence of Bacteroides.

CHAPTER 4

ANTIMICROBIAL SUSCEPTIBILITIES OF *BACTEROIDES* SPECIES

INTRODUCTION

The importance of anaerobes in human clinical infections has been increasingly recognised in the last decade. Improved methodology has resulted in an increased frequency of isolation from infections (Chow and Guze, 1974; Finegold, 1977). Changing antibiotic resistance patterns and the development of new drugs have necessitated the continual updating of antimicrobial susceptibility data.

The purpose of this study was two-fold. Firstly, it was designed to examine the *in vitro* activity of a wide selection of antibiotics against *Bacteroides* species. Secondly, it sought to provide susceptibility data for strains of known plasmid composition with the ultimate aim of determining any correlation between the plasmid presence and antibiotic susceptibility.

MATERIALS AND METHODS

Test Strains

All strains in the *Bacteroides* collection (Appendix 1) were tested together with the following reference strains: *B. fragilis* NCTC 9343, NTCC 9344 and NCTC 11870; *E. coli* NCTC 10418; *P. aeruginosa* NCTC 10662; *Staph. aureus* (Oxford) NCTC 6571; *C. perfringens* NCTC 8237 (Table 4.1).

Antimicrobial Agents

Antimicrobial agents were supplied as standard powders of known potency: benzylpenicillin and cefuroxime sodium (Glaxo), cefotaxime (claforan) : Roussel Laboratories Ltd.), moxalactam [(latamoxef disodium) Eli Lilly and Company Ltd.]; cefoxitin sodium and imipenem [(N-formimidoyl thienamycin monohydrate) Merck, Sharp and Dohme]; cefotetan disodium (Imperial Chemical Industries); chloramphenicol (Parke Davis and Company); clindamycin (Upjohn Ltd.); metronidazole (May and Baker Ltd.); erythromycin [(erythrocin I.V. lactobionate) Abbott Laboratories Ltd.] and nalidixic acid (Sigma Chemical Company).

Preparation of Antibiotic Solutions

Stock solutions of concentration 12.8 g/l (metronidazole), 20 g/l (erythromycin) and 100 g/l (all other antibiotics except chloramphenicol and nalidixic acid) were prepared by the addition of

TABLE 4.1 The minimum inhibitory concentrations of NCTC reference strains

Antimicrobial agent	NCTC Reference strain	Minimum Inhibitory Concentration mg/l	Literature reference
Pencillin	10418	8	Phillips and Williams (1978)
	6571	0.01	
Cefuroxime	9343	8	"
	10418	0.12	"
Cefoxitin	9343	4	"
	10418	1	"
Clindamycin	9343	0.25	"
	6571	0.06	"
Chloramphenicol	6571	4	"
	10418	1	"
Erythromycin	10418	16	"
	6571	0.12	"
Nalidixic acid	10418	1	"
Cefotaxime	10662	8	Personal communication Dr George, Eli Lilly and Co., Ltd.[1]
Moxalactam	10662	4	"
Cefotetan	11870	16	Personal communication P.J. Turner, I.C.I.[2]
Metronidazole	8237	4	Freeman et al. (1968)
Imipenem	9344	0.12	Eley and Greenwood (1986b)

1 Eli Lilly and Co Ltd, City Wall House, Basing View, Basingstoke, Hampshire.

2 I.C.I. = Imperial Chemical Industries, Mereside Alderly Park, Macclesfield Cheshire

sterile, distilled water to weighed quantities of the antibiotics. Stock solutions of chloramphenicol, concentration 100 g/l, were prepared in ethanol. Nalidixic acid stock solution of concentration 12.8 g/l was prepared in 2% NaOH. These solutions were divided into aliquots and stored at -70°C.

Before use the concentrations of the stock solutions were verified by broth dilution MIC tests against the reference strains. Tests were performed in 76 x 12 mm glass tubes with 1 ml volumes of a range of doubling dilutions suitable for each strain and each antibiotic solution. The range started three dilutions above the accepted MIC for the strain (Table 4.2) and ended three below. Aliquots of stock solution were first diluted with sterile distilled water or with ethanol (ethanol was used as a diluent for chloramphenicol until the final concentration required was less than 2.5 g/l) to a concentratin of 20 g/l (excepting metronidazole which was at a concentration of 12.8 g/l), and then further diluted in distilled water by the modified method of Ericsson and Sherris (1979) (Table 4.2) to 100 times the highest concentration required for the determination of each MIC. Further dilutions were made in Mueller-Hinton broth (Difco Laboratories). An inoculum of one drop (0.03 ml) of a 1 in 200 dilution of overnight culture of the reference strains was added to each tube. Two control tubes, each containing 1 ml Mueller-Hinton broth, were also prepared. The first, the growth control, was inoculated with the test strain; the second tube, the media control, was included as a sterility check. Incubation was anaerobically at 37°C for 24 h except where reference strains were aerobic or facultatively anaerobic. These strains were

TABLE 4.2 A system for preparing antibiotic dilutions for the determination of minimum inhibitory concentrations

Intermediate concentration mg/l	Directions for preparing intermediate solutions	Final concentration[2] mg/l
12800	6.4 ml 20000 mg/l + 3.6 ml sterile diluent[3]	128
6400	2 ml 12800 mg/ml (above) + 2 " " "	64
3200	1 " " " " + 3 " " "	32
1600	0.5 " " " " + 3.5 " " "	16
800	0.5 " " " " + 7.5 " " "	8
400	2 ml 800 mg/ml (above) + 2 " " "	4
200	1 " " " " + 3 " " "	2
100	0.5 " " " " + 3.5 " " "	1
50	0.5 " " " " + 7.5 " " "	0.5
25	2 ml 50 mg/ml (above) + 2 " " "	0.25
12.5	1 " " " " + 3 " " "	0.125
6.3	0.5 " " " " + 3.5 " " "	0.063
3.2	0.5 " " " " + 7.5 " " "	0.032
1.6 etc	2 ml 3.2 mg/ml (above) + 2 " " "	0.016

1 Modified method of Ericsson and Sherris (1979).
2 Final concentration is based on a dilution of 1 ml of intermediate solution with 90 ml agar and 9 ml blood.
3 The appropriate diluent was water or ethanol.

incubated under aerobic conditions. The MIC of each strain was recorded as the concentration of antibiotic at which there was no visible turbidity.

Determination of MICs

MICs of the test strains were determined by an agar dilution method (F. Brown, personal communication). The medium used was diagnostic sensitivity test agar [(D.S.T.) Oxoid] with nine per cent horse blood. Antibiotic solutions were diluted in distilled water to 100 times the required concentration by the modified method of Ericsson and Sherris [(1979) Personal communication, Table 4.2]. To 90 ml molten agar, cooled to 55°C, 9 ml of horse blood and 1 ml antibiotic solution were added. The inoculum was prepared from 24 h PPY cultures of the strains. *B. fragilis* group strains were diluted 1 in 100 in pre-reduced PPY while non-*B. fragilis* group strains were diluted 1 in 10. Cultures of test strains which grew poorly and gave no visible turbidity were not diluted.

Reference strains (Table 4.1) were included on each plate as controls. Where two strains were available both were used. Inoculation was with a multipoint inoculator (Mast Laboratories), the inoculum size being ca 0.03 ml.* Incubation was at 37°C for 40 h in an anaerobic atmosphere (as previously outlined). Plates containing cefotaxime and moxalactam were further incubated overnight at 37°C in an aerobic atmosphere to facilitate growth of the standard strain *P. aeruginosa* NCTC 10662.

* (ca 10^5 organisms)

The range of final concentrations tested for each antibiotic (mg/l) was: imipenem (0.016 - 8); metronidazole, chloramphenicol (0.063 - 32); clindamycin (0.016 - 128); cefoxitin, moxalactam, cefotaxime, cefuroxime, cefotetan, penicillin, erythromycin and nalidixic acid (0.25 - 128). The MIC for each strain was the antibiotic concentration at which there was no visible growth or the growth was reduced to a faint haze with no distinct colonies visible. The recorded MICs of the test strains were adjusted to take account of any discrepancies between the experimental and literature values of the MICs of reference strains.

Additional Tests for Metronidazole Resistant Strains

Further tests were conducted on metronidazole-resistant strains to ensure that they were Bacteroides species.

Growth under microaerophillic conditions

The strains were streak-plated on to blood agar plates incubated anaerobically at 37°C for 48 h and at 37°C for seven days in 10% carbon dioxide plus air (CO_2 incubator).

Gas-liquid chromatography

GLC profiles were obtained for each strain using the method outlined in Chapter 2.

RESULTS

All strains from the melaninogenicus/oralis and asaccharolytic groups were highly sensitive to chloramphenicol and clindamycin (Tables 4.3 and 4.4). The MIC90 for both groups was less than 0.25 mg/l for clindamycin and less than 2 mg/l for chloramphenicol. All the B. fragilis group strains were sensitive to chloramphenicol but 8% were resistant to clindamycin (Table 4.5). Resistance to erythromycin was found in all three groups. Of the melaninogenicus/oralis and asaccharolytic strains, 2.4% and 4.6% respectively were resistant, but the MIC90 for these strains, 1 mg/l, was a third of the maximum achievable serum level for this antibiotic. With the B. fragilis group, however, 87.5% of the strains were resistant to erythromycin and the MIC90 was 32 mg/l (Table 4.6).

Metronidazole was active against all three groups of Bacteroides. However with three strains, one B. melaninogenicus and two B. fragilis strains, the MIC was >16 mg/l the maximum achievable serum level of this antibiotic (Tables 4.3 - 4.5). These strains grew on blood agar incubated anaerobically at 37^{o}C but not on blood agar incubated in an atmosphere of 10% carbon dioxide in air. Their GLC profiles of short chain fatty acid and products of glucose metabolism, and results of a range of phenotypic tests confirmed the identities of these isolates. All bacteroides strains examined were resistant to nalidixic acid.

TABLE 4.3 Susceptibility of the melaninogenicus/oralis group to non-beta-lactam antimicrobial agents.

Antimicrobial agent	Cumulative percentage susceptible to concentration (mg/1)									
	<0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	>128.0
Metronidazole (16)*	2.4	19.5	48.8	80.5	97.6		100			
Chloramphenicol (16)*		26.8	85.4	95.1	100					
Clindamycin (8)*	92.7	97.6	100							
Erythromycin (3)*	24.3	63.4	87.8	97.6			100			

* Maximum achievable serum concentrations.

TABLE 4.4 Susceptibility of the asaccharolytic Bacteroides to non-beta-lactam anti-microbial agents.

Antimicrobial agent	Cumulative percentage susceptible to concentration (mg/1)									
	<0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	>128.0
Metronidazole (16)*	54.5	68.2	90.9	100						
Chloramphenicol (16)*	9.1	50.0	86.4	95.5	100					
Clindamycin (8)*	100									
Erythromycin (3)*	63.6	86.3	95.4			100				

* Maximum achievable serum concentrations.

TABLE 4.5 Susceptibility of the Bacteroides fragilis group to non-beta-lactam anti-microbial agents.

Antimicrobial agent	Cumulative percentage susceptible to concentration (mg/l)									
	<0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	>128.0
Metronidazole (16)*	8.0	22.7	44.3	79.5	96.6		98.9	100		
Chloramphenicol (16)*			2.3	18.2	95.5	100				
Clindamycin (8)*	75.0	87.5	89.8	92.0			93.2			100
Erythromycin (3)*				12.5	36.4	63.6	75.0	90.9		100

* Maximum achievable serum concentrations.

TABLE 4.6 Comparative susceptibility of Bacteroides groups to non-beta-lactam antimicrobial agents

Bacteroides Group (number of isolates)	Antimicrobial Agent	MIC* (mg/l) MIC50	MIC90
fragilis (88)	Metronidazole	>1.0<2.0	>2.0<4.0
	Clindamycin	<0.25	1.0
	Chloramphenicol	>2.0<4.0	>2.0<4.0
	Erythromycin	>4.0<8.0	32.0
melaninogenicus/ oralis (41)	Metronidazole		
	Clindamycin	<0.25	<0.25
	Chloramphenicol	>0.5<1.0	>1.0<2.0
	Erythromycin	>0.25<0.5	>1.0<2.0
asaccharolytic (22)	Metronidazole	<0.25	1.0
	Clindamycin	<0.25	<0.25
	Chloramphenicol	0.5	>1.0<2.0
	Erythromycin	<0.25	>0.25<1.0

* Minimum inhibitory concentration

The asaccharolytic Bacteroides were sensitive to all the beta-lactam antibiotics except cefotetan to which 4.6% of the strains were resistant (Table 4.7). Imipenem, moxalactam, cefoxitin and cefotaxime and cefuroxime all showed excellent activity against the melaninogenicus/oralis strains. Low levels of resistance, 2.4%, were displayed to penicillin (2.4%) and cefotetan (7.3%) (Table 4.8, Fig. 4.1).

Imipenem was the only beta-lactam antibiotic to which all the strains of the B. fragilis group were sensitive (Table 4.9). Resistance varied from 1.1% to cefoxitin and moxalactam, to 50.0% to penicillin.

Within the Bacteroides fragilis group there was variation in the susceptibility to cefotetan (Table 4.10, Fig. 4.2). The MIC90 for the B. fragilis species were less than 32 mg/l, the peak serum level for cefotetan, and only 8.8% of the strains were resistant to cefotetan concentrations above this level. For the other members of the B. fragilis group the MIC90 was between 64 mg/l and 128 mg/l. Resistance to cefotetan was found in 84.2% of these strains. This wide variation was not observed with the other cefamycins investigated, although the B. fragilis species were more sensitive than the other species to both cefoxitin and moxalactam (Table 4.10).

TABLE 4.7 Susceptibility of the asaccharolytic Bacteroides to beta-lactam antimicrobial agents.

Antimicrobial agent	Cumulative percentage susceptible to concentration (mg/l)									
	<0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	>128.0
Penicillin (20)*	90.9			95.4		100				
Cefuroxime (32)*	54.5	68.1	77.2	86.3	90.8	95.3	100			
Cefotaxime (16)*	72.7	81.8	86.3	90.8	95.3		100			
Cefotetan (32)*	40.9	59.1	86.4			90.9	95.4		100	
Cefoxitin (32)*	72.7	81.8	86.3	90.8	95.3	100				
Moxalactam (32)*	72.7	95.4				100				
Impipenem (32)*	81.8	95.4		100						

* Maximum achievable serum concentrations.

TABLE 4.8 Susceptibility of the melaninogenicus/oralis group to beta-lactam anti-microbial agents.

Antimicrobial agent	Cumulative percentage susceptible to concentration (mg/l)									
	<0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	>128.0
Penicillin (20)*	17.1	29.3	46.3	73.2	85.4	90.2	97.6	100		
Cefuroxime (32)*	12.2	19.5	29.3	53.7	80.5		87.8	100		
Cefotaxime (16)*	46.3	63.4	68.3	80.5	95.1	100				
Cefotetan (32)*			7.3	12.2	31.7	63.4	82.9	92.7	100	
Cefoxitin (32)*	39.0	63.4	73.2	87.8	95.1	97.6	100			
Moxalactam (32)*	22.0	31.7	63.4	70.7	92.7	97.6	100			
Imipenem (32)*	90.2	95.1		100						

* Maximum achievable serum concentrations.

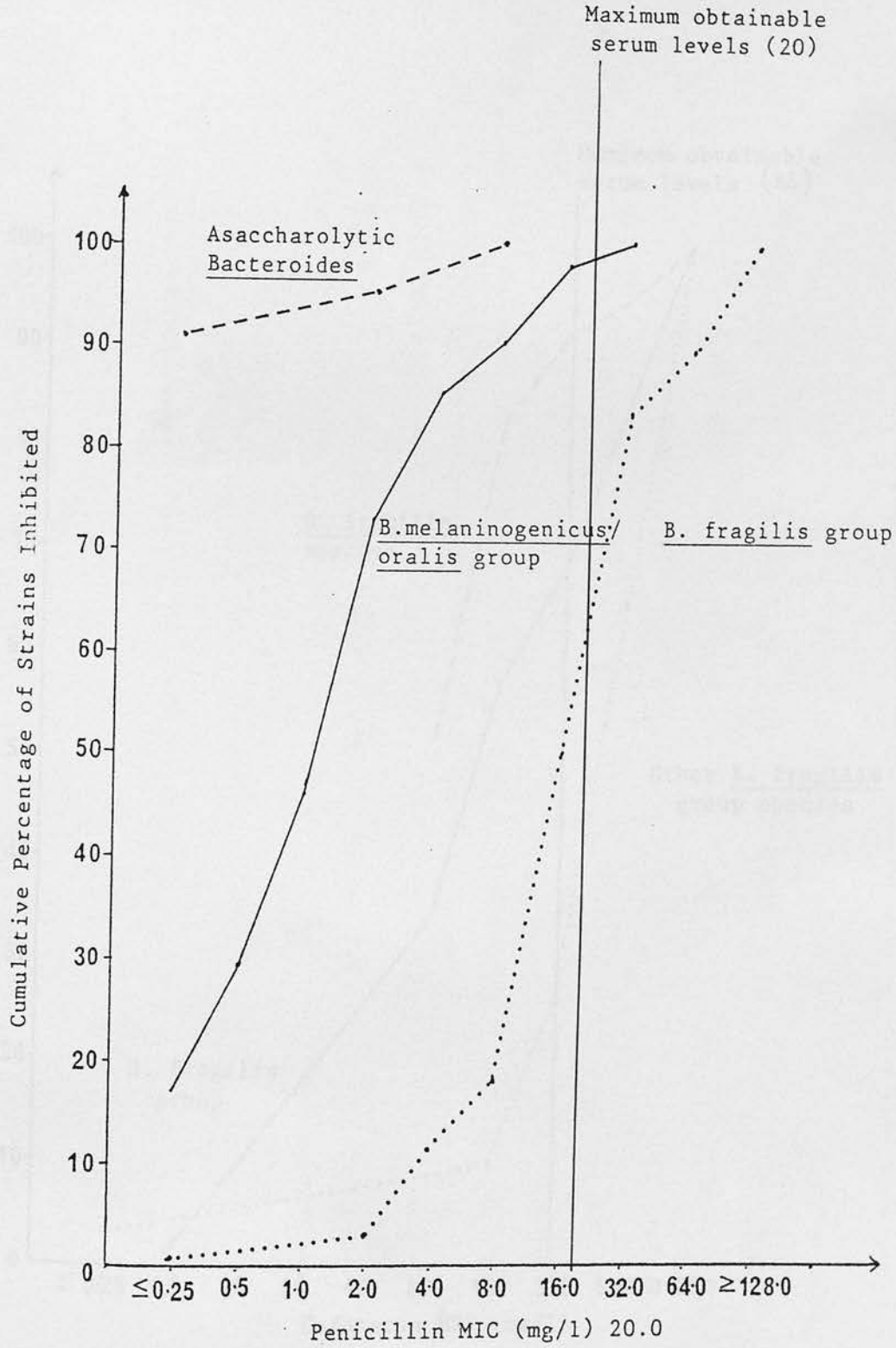


Figure 4.1

The susceptibility of Bacteroides to penicillin as determined by an agar dilution method.

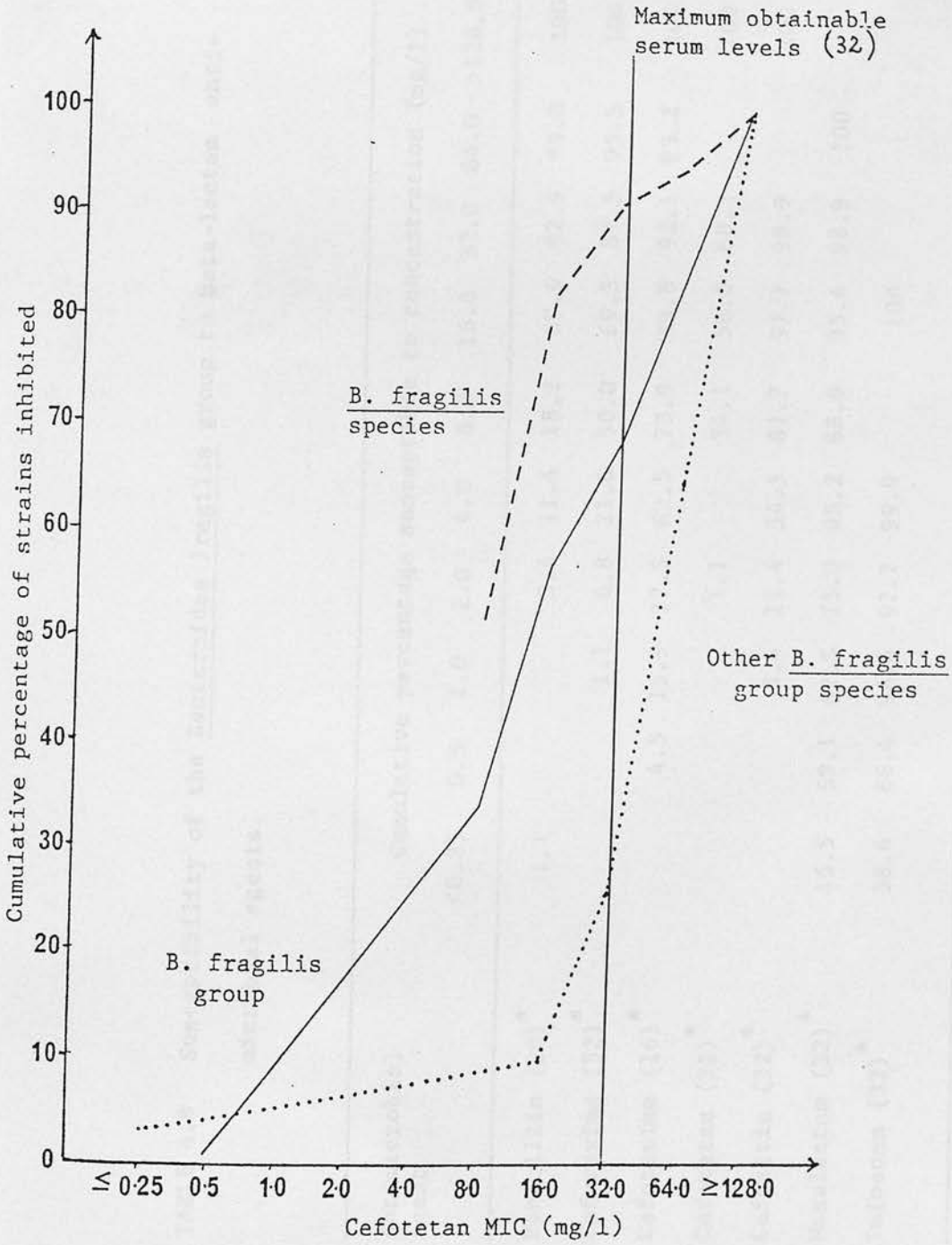


Figure 4.2

The susceptibility of the Bacteroides fragilis group to cefotetan as determined by an agar dilution method.

TABLE 4.9 Susceptibility of the Bacteroides fragilis group to beta-lactam antimicrobial agents.

Antimicrobial agent	Cumulative percentage susceptible to concentration (mg/l)									
	<0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	>128.0
Penicillin (20)*	1.1			3.4	11.4	18.2	50.0	82.9	89.8	100
Cefuroxime (32)*			1.1	6.8	21.6	50.0	69.3	87.5	95.5	100
Cefotaxime (16)*		4.5	15.9	37.5	62.5	73.9	89.8	92.1	93.2	100
Cefotetan (32)*				1.1		34.1	56.8	68.2		100
Cefoxitin (32)*			3.4	11.4	54.5	81.7	97.7	98.9		100
Moxalactam (32)*	45.5	59.1	62.5	75.0	85.2	88.6	95.6	98.9	100	
Imipenem (32)*	38.6	86.4	96.6	97.7	99.9		100			

* Maximum achievable serum concentrations.

TABLE 4.10 Comparative susceptibility of the Bacteroides fragilis group to the cephamycins.

(number of isolates)	Antimicrobial Agent	MIC* (mg/l) MIC50	MIC90
Bacteroides fragilis group (88)	Cefotetan	>8.0<16.0	>64.0<128.0
	Cefoxitin	>2.0<4.0	>8.0<16.0
	Moxalactam	>0.25<0.5	>8.0<16.0
Bacteroides fragilis (57)	Cefotetan	<8.0	>16.0<32.0
	Cefoxitin	>2.0<4.0	>4.0<8.0
	Moxalactam	<0.25	>2<4
Other (31)	Cefotetan	>32.0<64.0	>64.0<128.0
	Cefoxitin	>4.0<8.0	>8.0<16.0
	Moxalactam	>2.0<4.0	>8.0<16.0

DISCUSSION

The agar dilution method used for this study has been shown to give reliable and reproducible results (Sutter et al., 1979). This method was selected instead of the disk diffusion method because of the number of bacterial strains and antibiotics involved.

A range of beta-lactam and non-beta-lactam antibiotics was chosen in order to give a balanced perspective of the action of antimicrobial agents against the bacteroides. Chloramphenicol, clindamycin and metronidazole are non-beta-lactam antibiotics known to have high activity against Bacteroides species (Brown and Waatti, 1980; Cuchural et al., 1981; Nastro and Finegold, 1972; Snydman et al., 1980; Sutter and Finegold, 1976). Erythromycin was studied because its resistance is often associated with resistance to clindamycin (Guiney et al., 1983).

Nalidixic acid is a quinolone antibiotic which is mainly active against Gram-negative aerobes (Garrod et al., 1981). Bacteroides species are resistant to nalidixic acid due to the impermeability of the drug (Watt, 1979). Crumplin and Smith (1981) demonstrated that R factors (plasmids) in Escherichia coli may increase the susceptibility of their bacterial host strain to the bactericidal effects of nalidixic acid. The activity of this antimicrobial agent against Bacteroides was studied as no parallel investigation has been undertaken with these species.

The non-B. fragilis groups of Bacteroides are known to be more susceptible to antimicrobial agents than the B. fragilis group

(Wexler and Finegold, 1987). The results of this study are in agreement with these findings. The asaccharolytic group were found to be the most sensitive, but due to the varying styles of reporting in the literature, it is difficult to make a direct comparison (asaccharolytic bacteroides are often grouped with other species as in Table 1.3).

The high activity of chloramphenicol against the Bacteroides was expected as resistance to chloramphenicol is very rare (Martinez-Suarez et al., 1985; Phillips et al., 1981). Levels of clindamycin resistance range from 2 to 20% of all strains reported in the literature (Acar et al., 1981; Brown and Waatti, 1980). The results of this study are similar to those of Cuchural et al. (1981) who found clindamycin resistance in 7% of all strains examined. Erythromycin, though mainly active against Gram-positive species, has been shown to have some activity against Bacteroides spp. However, the emergence of resistant strains has greatly resitricted its use (Finegold et al., 1972) and in this study over 80% of B. fragilis group isolates were resistant to erythromycin.

Low level resistance to erythromycin and clindamycin (resistance to 5 - 20 mg/l) has been described as less prevalent among Bacteroides than high level resistance (resistance to greater than 64 mg/l) (Guiney et al., 1983). While only one strain displayed low level resistance to clindamycin, this type of resistance to erythromycin was the only one displayed by the melaninogenicus/oralis and asaccharolytic groups of Bacteroides. Among the B. fragilis group, of 87.5% of strains resistant to erythromycin, 62.5% showed low level resistance, 9% high level resistance and 15.9%

resistance between the two. These results indicate a greater incidence of low level resistance to erythromycin than previously reported.

Although high level resistance to erythromycin is usually accompanied by resistance to clindamycin and the streptogrammins (MLS resistance) (Guiney et al., 1983; Privatera et al., 1981) only seven of nine strains showing high level resistance to erythromycin showed high level resistance to clindamycin. This indicates the existence of another mechanism of resistance to erythromycin besides MLS resistance. All strains with high level resistance to clindamycin showed high level resistance to erythromycin. Low level resistance to erythromycin is accompanied by varying levels of resistance to clindamycin and the streptogrammins. None of the strains resistant to low levels of erythromycin showed cross-resistance to clindamycin.

Despite reports from other countries of resistance to metronidazole (Acar et al., 1981; Appelbaum and Chatterton, 1978; Chow et al., 1975) such reports are rare within the United Kingdom. Apparent resistance in sensitive strains may be due to inoculum size or inadequate conditions of anaerobiosis (Rahimi et al., 1974; Tally et al., 1978). In the method used in this study, the inoculum size was standardised for all the strains and the anaerobic techniques were the well established methods of Collee et al. (1972). These factors were therefore unlikely to be the reason for the resistance to metronidazole found in three strains. Growth of these strains in an anaerobic atmosphere but not in an atmosphere of carbon dioxide

plus air indicated that the strains were anaerobes, and other tests confirmed that they were Bacteroides.

Within the UK there have been no confirmed reports of resistance in non-fragilis groups. Such reports outside the UK appear also to lack independent corroboration (Acar et al., 1981). Ingham et al., (1978) showed the development of resistance to metronidazole, in a strain of B. fragilis following prolonged treatment with this antimicrobial agent. All three strains were originally supplied by clinicians and while background information is limited it is known that the resistant strain B. melaninogenicus MPRL 528 was isolated from an undrained brain abscess following metronidazole treatment. It is impossible to state whether this resistance is similar to that observed by Ingham et al. (1978) as the duration of treatment is unknown.

The effect of penicillin and the cephalosporins against Bacteroides is known to be limited and to vary between the groups. The B. fragilis group show intrinsic resistance to penicillin (Tally et al., 1979; Acar et al., 1981) possibly by a non-beta-lactamase mechanism as the beta-lactamases of this group are predominantly cephalosporinase in nature (Anderson and Sykes, 1973; Britz and Wilkinson, 1978; Timewell et al., 1981b; Maskell et al., 1984). High levels of resistance such as that observed in this study have been reported by other workers (Tally et al., 1979; Acar et al., 1981). It is, however, difficult to assess from the literature the percentage of isolates resistant to penicillin due to differences in styles of reporting and MIC breakpoint (4 - 20 mg/l) (Watt, 1979; Brown and Waatti, 1980; Wexler and Finegold, 1987).

Sensitivity among the non-fragilis groups has been widely reported (Sutter and Finegold, 1976; Finegold, 1977; Watt, 1979) but the MIC90 reported in the literature for these strains (Table 1.3) was higher than that observed in this study (Table 4.3). The beta-lactamase enzymes of the non-fragilis groups have mainly penicillinase activity and resistance to penicillin has been known to occur (Murray and Rosenblatt, 1977; Salyers et al., 1977; Timewell et al., 1981a). All asaccharolytic strains examined were sensitive to penicillin but a small percentage of the melaninogenicus/oralis group were resistant. Resistance by a small proportion of melaninogenicus/oralis strains has been similarly observed by Ohm-Smith et al. (1982). While all resistant non-fragilis group strains were of the B. bivius species, this study did not confirm the results of Snyderman et al. (1980) who found that approximately 50% of B. bivius and B. disiens strains were resistant to penicillin.

Darland and Birnbaum (1977) found that more than 50% of B. fragilis isolates were resistant to cefuroxime and 20 - 50% of the B. fragilis isolates reported in the literature were resistant to cefotaxime (Cuchural et al., 1981; Fu and Neu, 1981; Phillips et al., 1981; Wexler and Finegold, 1987). Among the non-fragilis group 4% resistance to cefotaxime was observed by Wexler et al. (1987). The results of this study are low by comparison and no significant difference was found in the activity of cefuroxime, a second generation cephalosporin and cefotaxime, a third generation cephalosporin. This absence of increased activity of cefotaxime relative to cefuroxime was also shown by Jorgensen et al. (1980). While this may indicate continued susceptibility of the third

generation cephalosporins to the beta-lactamase enzymes of the B. fragilis group, other factors could also be involved. The relative hydrolysis rate of cefotaxime (28 - 54%) is less than that of cefuroxime (58 - 75%) (Fu and Neu, 1981; King et al., 1980; Schrinner et al., 1980). If beta-lactamase activity was the only factor involved then cefotaxime should have been more active than cefuroxime. This was shown to be the case with the non-fragilis groups - but since the beta-lactamases of these groups have mainly penicillinase activity, beta-lactamase resistance may not be the sole factor responsible.

Reports vary on the level of activity of the cefamycins. The literature data summarised in Table 1.3 show that the results vary both within and between groups. All cefamycins have been shown to be inhibitors of beta-lactamse enzymes (Darland and Birnbaum, 1977; Fu and Neu, 1981; Grassi et al., 1983).

Moxalactam has been shown by Fu and Neu (1981) to be superior to cefoxitin in beta-lactamase inhibition. This was thought to be due to the carboxyl group at position 10 since this group in carbenicillin appeared to confer beta-lactamase stability and inhibitory activity.

Investigations have shown that of the three cefamycins examined during this study, moxalactam is the most active compound (Clarke and Zemcou, 1983; Moosdeen et al;, 1983) although there is some evidence that cefoxitin has superior activity against some species of the B. fragilis group (Eley and Greenwood, 1984; Ruckdeschel, 1983).

The activity of cefotetan was shown in general to be less than that of cefoxitin (Clarke and Zemcou, 1983; Watt and Brown, 1985; Werner 1983).

In this study, cefoxitin and moxalactam had identical levels of activity while cefotetan was much less active. While only 1.1% of the B. fragilis group strains were resistant to cefoxitin and moxalactam, 31.8% of these strains were resistant to cefotetan. Resistance to cefotetan was also observed among a small percentage of the melaninogenicus/oralis and asaccharolytic strains. Reports of resistance in non-fragilis groups are rare. The MIC90 of these strains reported in the literature is less than 32 mg/l (Table 1.3). However, Watt and Brown (1985) similarly observed resistance in non-fragilis strains.

Variable results of activity of cefotetan in Bacteroides have been reported in the literature. Grassi et al. (1983) showed that approximately 50% of B. fragilis strains were resistant to cefotetan. This is in contrast to the results of Watt and Brown (1985) who showed that 90% of B. fragilis strains were inhibited by readily available serum concentrations of cefotetan. This discrepancy may be due in part to the number of isolates examined. Watt and Brown (1985) investigated the susceptibility to cefotetan of 120 strains of B. fragilis as opposed to the 11 strains investigated by Grassi et al. (1983).

The bimodal distribution of MICs to cefotetan within the B. fragilis groups, reported by Grassi et al. (1983), was not observed. The B. fragilis species were found to be far more

susceptible than the other members of the group. These results confirm those of other workers (Moosdeen et al., 1983; Ruckdeschel, 1983; Watt and Brown, 1985; Werner, 1983) and re-emphasises the need for speciation within the group (Wexler and Finegold, 1987). Less than 10% of B. fragilis strains were found to be resistant to cefotetan but this species is responsible for more than 60% of all anaerobic infections (Gorbach and Bartlett, 1974 a and b; Mancini and Behme, 1977). Despite the fact, therefore, that other B. fragilis group strains are resistant to cefotetan, this antibiotic can be used effectively in the treatment of bacteroides infections if the infecting organisms are B. fragilis and melaninogenicus/oralis and asaccharolytic Bacteroides group strains.

The excellent activity of imipenem (N-formimidoyl thienamycin) against Bacteroides spp. is thought to be due to its high resistance to beta-lactamase activity (Brown et al., 1981; Hanslo et al., 1981; Labia et al., 1986). This antibiotic is not in use in Great Britain but was of interest as a prospective therapeutic agent for bacteroides infection. These results are in agreement with those of other workers and show a high degree of sensitivity even among the more resistant B. fragilis group (Hoffler, 1986; Tally and Jacobus, 1983; Wexler and Finegold, 1987).

CHAPTER 5

CORRELATION STUDIES OF ANTIBIOTIC RESISTANCE, PLASMID PROFILES AND OUTER MEMBRANE PROTEIN COMPONENTS OF *BACTERIODES*

INTRODUCTION

Several groups of workers have attempted to correlate the presence of plasmids with phenotypic properties important in the expression of antibiotic resistance and virulence (Beul et al., 1985; Mays et al., 1982; Riley and Mee, 1984; Stiffler et al., 1974; Tinnell and Macrina, 1976; Wallace et al., 1981). While most of these attempts have failed, a correlation has been established between plasmid presence and resistance to the macrolide-lincosamide-streptogramin (MLS) antibiotics and to tetracycline.

Much evidence indicates the existence of a single, high-level resistance (resistance to > 64mg/l) determinant to MLS antibiotics which is widely disseminated among Bacteroides species (Guiney et al., 1984; Privatera et al., 1979; Rotimi et al., 1981; Smith and Macrina, 1984; Tally et al., 1979; Welch et al., 1979). This study was designed to investigate the relationship of plasmid DNA to observed macrolide-licosamide resistance and to determine possible homology between a high-level resistance determinant described in the literature and the determinants on the DNA of our resistant isolates.

Cefoxitin has been widely used in the treatment of mixed surgical and gynaecological infections as it is active against both facultative organisms and strict anaerobes. While some reports show that cefoxitin resistant strains of the B. fragilis group appear only sporadically and in low frequencies (Cuchural et al., 1983; Olsson et al., 1977) other reports show an incidence of cefoxitin resistance varying between 8 - 30% (Bieluch et al., 1987; Cuchural et al., 1984; Tally et al., 1983). Several mechanisms of resistance have

been postulated. These include hydrolysis by beta-lactamases (Cuchural et al., 1986a, 1986b; Yotsuji et al., 1983) and alterations in cell wall structure (Cuchural et al., 1983; Dornbusch et al., 1980).

Piddock and Wise (1987) induced mutation in a sensitive strain of B. fragilis, B1 (MIC of 4 mg/l to cefoxitin) by the use of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG). They isolated four mutants, B265, B266, B269 (MIC of 16 mg/l to cefoxitin) and B271 (MIC of 32 mg/l to cefoxitin). The mechanism of cefoxitin resistance in these five strains as well as a number of naturally occurring resistant strains isolated by Dornbusch et al. (1980) and Dr Francis Tally (Tufts University School of Medicine, New England Medical Centre, Boston, Mass, USA) was investigated.

A comparison was made of the outer membrane protein (OMP) profile of the mutants and parent strains with that of a standard strain, B. fragilis NCTC 9343. NCTC 9343 had been previously determined by Piddock and Wise (1986) to be identical to B. fragilis B1 in penicillin binding protein (PBP) competition assay against 34 beta-lactam antibiotics. Piddock and Wise (1987) found that the OMP profiles of the mutants were identical to that of the parent strain and to that of NCTC 9343 and concluded that resistance to cefoxitin in these strains was attributable to altered expression of outer membrane proteins.

Correlations were found between changes in the affinity of PBP1 and PBP2 of three of the mutants (B265, B269 and B271) and decreased susceptibility to cefoxitin. The mechanism of resistance in the

fourth mutant remained unknown. Differences in the OMP profile of NCTC reference strains compared with some of the naturally resistant strains were demonstrated. Alterations in relative PBP profiles were in some cases also observed. Based on these findings Piddock and Wise (1987) postulated that there were two additional mechanisms of cefoxitin resistance in bacteroides; alteration in PBP profile and alteration in porin proteins.

The genetic basis of resistance in the strains was not examined. Despite the fact that the use of NTG can result in multiple mutations (Guerola et al., 1971), in the absence of significant cross-resistance to other antibiotics Piddock and Wise (1987) assumed that mutation was in a single gene conferring cefoxitin resistance. A number of the strains were obtained and the role of plasmid DNA in cefoxitin resistance examined.

(i) Curing of Clindamycin and Erythromycin Resistant Strains

Bacterial Strains

Curing of twelve Bacteroides strains resistant to clindamycin and/or erythromycin was attempted (Table 5.1 and Fig 5.1).

Curing Experiments

Curing experiments were conducted by the modified method of Rotimi and Duerden (1982) with the curing agent eithidium bromide.

Determination of subinhibitory concentration of ethidium bromide

The subinhibitory concentration of ethidium bromide was determined in a preliminary study using B. fragilis strain V479. The number of cells per ml was determined by direct counting of cells from overnight cultures using a Thoma ruled cell count chamber with 0.1 mm depth (Hawksley, England). Bacterial cells (200 - 400) were counted and the average number calculated using the formula:

$$\text{Number of cells/ml} = \text{Number of cells per square} \times \text{dilution factor} \times 4 \times 10^6$$

Seven determinations were made and based on these results a standard procedure for the dilution of overnight cultures to a final concentration of ca 10^5 cells/ml was devised.

TABLE 5.1 Minimum Inhibitory Concentrations (MIC) of Bacterial Strains Resistant to Clindamycin and/or Erythromycin

Strains	MIC Clindamycin mg/l	MIC Erythromycin mg/l	Plasmid
B. fragilis V479	>64	>64	+
B. fragilis MH2	>64	>64	+
B. fragilis MH11	16	8	+
B. fragilis MH12	>64	>64	+
B. fragilis MH48	<0.25	64	-
B. fragilis MH67	0.50	16	-
B. fragilis MH68	16	>64	+
B. uniformis MH29	>64	>64	-
B. uniformis MH58	16	>64	-
B. uniformis M59	16	>64	-
B. thetaiotaomicron MH79	>64	>64	+/-

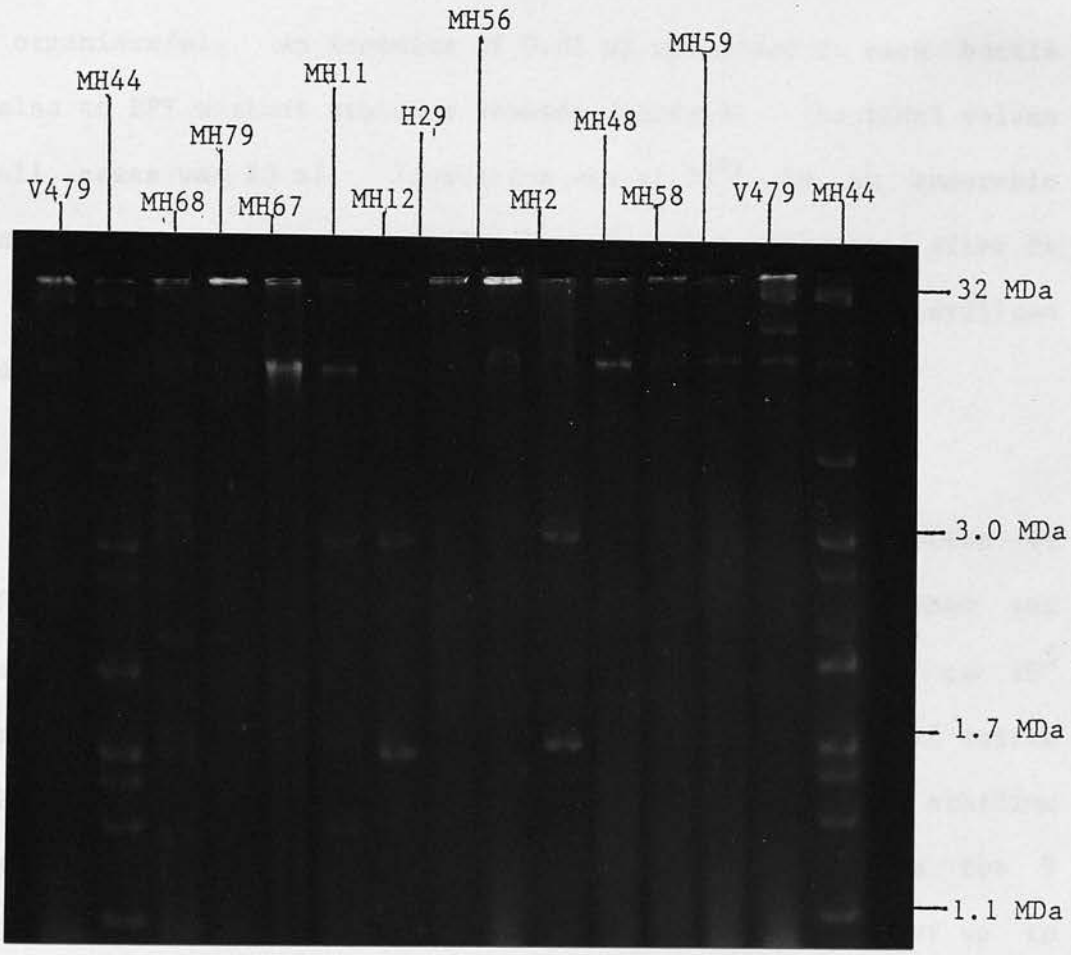


Figure 5.1

Agarose gel (0.6%) showing plasmid profile of bacteroides strains resistant to clindamycin and/or erythromycin.

Ethidium bromide was added to PPY complete in universal bottles to give final concentrations of 8, 16, 20, 24 and 32 mg/l and the media autoclaved at 121°C for 15 min. Cells of V479 were pelleted by centrifugation at 2,500 g for 20 min at room temperature. They were washed and resuspended in PBS (pH 7.2) to a final density of ca 10^5 organisms/ml. An inoculum of 0.01 ml was added to each bottle and also to PPY without ethidium bromide (control). The final volume in all cases was 20 ml. Incubation was at 37°C in an anaerobic atmosphere (as previously described). Growth was observed after 24 and 48 h. The procedure was repeated using filter-sterilised ethidium bromide.

Curing of test strains

Cells of the test strains were pelleted by centrifugation at 2,500 g for 20 min at room temperature. They were washed and resuspended in PBS (pH 7.2) to a final concentration of ca 10^5 organisms/ml. An inoculum of 0.01 ml was added to a universal bottle of PPY (control) and PPY containing filter-sterilized ethidium bromide 20 mg/l. Incubation was in an anaerobic atmosphere for 5 days at 37°C. Serial dilutions of broth culture (0.1 ml) of up to 10^{-8} dilution were spread-plated on to blood agar and blood agar containing clindamycin 8 mg/l or erythromycin 8 mg/l. Growth was examined after incubation in an anaerobic atmosphere for 40 h at 37°C. Curing was determined by a comparison of growth on the non-selective (blood agar) and selective (blood agar with antibiotic) media. Organisms which grew on the non-selective but not on the selective media were considered cured.

(ii) DNA Homology Studies

Bacterial Strains

Plasmid DNA from E. coli strain V1476 (supplied by Professor Francis Macrina, Virgina Commonwealth University) containing the pVA1476 plasmid with the Hind III-Taq I fragment of erm f (the MLS resistance determinant of the 27 MDa pBF4 plasmid from strain V479) was used as the genetic probe. All bacterial strains resistant to clindamycin and erythromycin were investigated.

Preparation of Purified DNA

Purified plasmid and chromosomal DNA were prepared by the method previously described for the preparation of purified plasmid DNA (Chapter 3). Strains without plasmids had only one DNA band and this was harvested. Hybridization studies were with plasmid DNA in plasmid-containing strains and with chromosomal DNA in plasmid-free strains.

DNA Hybridization

The BluGENE™ Nonradioactive Nucleic Acid Detection System for the detection of DNA sequences in Southern-blot hybridization was used. The method was that outlined by the Bethesda Research Laboratories and involved the following: incorporation of biotinylated mucleotide into a DNA probe; hybridization of the biotin-labelled DNA probe to nucleic acid target sequences immobilized on a nitrocellulose membrane and colorimetric determination of the probe-target hybrid with the BluGENE™ streptavidin-alkaline phosphatase conjugate and dyes.

Transfer of DNA from Agarose Gels to Nitrocellulose Filters

Purified DNA separated on agarose gels was observed and photographed under ultra-violet as outlined in Chapter 3. Each gel contained DNA from three test strains plus DNA from B. fragilis V479 or E. coli V1476 which acted as the positive controls. Transfer to nitrocellulose filters was by the modified method of Maniatis et al. (1982) (Fig 5.2). The gel was transferred to a glass plate and unused areas trimmed away with a sterile scalpel. The plate was placed in a shallow plastic tray. DNA was denatured by soaking the gel in 0.25 M HCl for 30 min followed by soaking the gel twice for 30 min in 1.5 M NaCl and 0.5 M NaOH. The gel was neutralized by soaking twice in several volumes of a solution of 1 M Tris HCl (pH 8.0) and 1.5 M NaCl for 30 min. Denaturation and neutralization were at 4°C.

Whatman 3MM filter paper, paper towels and a piece of nitrocellulose filter paper were cut to the same dimensions of the gel. The nitrocellulose filter was soaked in 2 x SSC buffer (Appendix 4) until it was completely wet and layered carefully on top of the gel. All air bubbles trapped between the nitrocellulose and the gel were removed. A piece of Whatman 3MM paper was moistened and placed on top of the nitrocellulose with care again being taken to remove all air bubbles. A stack of Whatman 3 MM paper and a stack of paper towels was placed on top of them. A glass plate was put on top of the stacks and it was weighed down with a 500 g weight (Fig 5.2).

The transfer of DNA was allowed to proceed for 24 h and the filter then soaked in 6 x SSC at room temperature for 5 min. Excess

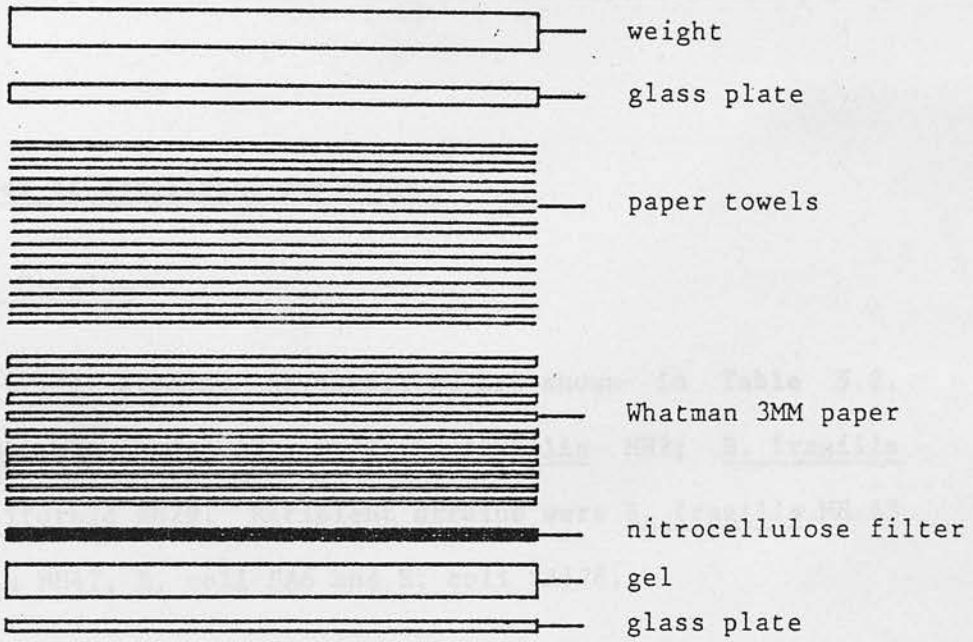


Figure 5.2

Method of transfer of DNA from agarose gels to nitrocellulose filter paper (modified from Maniatis et al., 1982).

fluid was allowed to drain and the filter dried at room temperature on a sheet of 3MM paper. The dried filter was then placed between two sheets of 3MM paper, baked for 2 h at 80°C under vacuum and then stored at room temperature, under vacuum between sheets of 3MM paper, until required.

(iii) Transfer of antibiotic resistance

Bacterial Strains

The bacterial strains investigated are shown in Table 5.2. Donor strains were *B. fragilis* V479; *B. fragilis* MH2; *B. fragilis* MH11 and *B. uniformis* MH29. Recipient strains were *B. fragilis* MH 43 and *B. fragilis* MH47, *E. coli* SA6 and *E. coli* SA128.

Selective Media

A combination of two antibiotics, one to which the donor strain was resistant and one to which the recipient strain was resistant, was used in the selective media. Antibiotic concentrations were as follows: penicillin, 32 mg/l; cefotaxime, 32 mg/l; clindamycin and erythromycin, 32 mg/l.

Transfer Experiments

The mating procedure was the modified method of Rotimi *et al.*, (1981). Twenty-four hour PPY cultures (5 ml) of donor and recipient strains were mixed and centrifuged at 2,500 g for 20 min at room temperature. The supernatant was decanted and 1 ml of prereduced PPY was carefully layered on top of the cell pellet. The mixture was incubated anaerobically at 37°C for 24 h and the cell pellet was then

TABLE 5.2 Bacterial Strains Used in Antibiotic Resistance Transfer Experiments

Bacteroides Species	Strain Number	Phenotype*	Plasmid Size (MDal)
B. fragilis	V479	Cc^r, Erm^r, PEN^s, CTX^s, CXM^s	27
B. fragilis	MH2	Cc^r, Erm^r, CTX^s	3.3
B. fragilis	MH11	Cc^r, Erm^r, CTX^s, CXM^s	3.2
B. fragilis	MH12	Cc^r, Erm^r, PEN^s, CTX^s, CXM^s	3.3, 1.5
B. fragilis	MH47	Cc^s, Erm^s, PEN^r, CTXr, CXM^r	None present
B. uniformis	MH43	Cc^s, Erm^s, PEN^r, CXM^r	None present
E. coli	SA6	Erm, MIC, 32 mg/l, Rif^r, Amp^s	None present
E. coli	SA128	Erm, MIC, 32 mg/l, Rif^r, Amp^s	None present

[1] Cc - clindamycin
Erm - erythromycin
PEN - penicillin
CTX - cefotaxime
CXM - cefuroxime
Rif - rifampicin
Amp - ampicillin

[2] ^{s} - sensitive to antibiotic
^{r} - resistant to antibiotic

resuspended. A sample (0.1 ml) was plated on selective media and incubated anaerobically for 24 - 48 h.

The inability of both donor and recipient strains to grow on the selective media was also tested. Broth cultures (10 ml) of donor and recipient strains were treated similarly to the mating mixtures and included as negative controls.

Further tests for E. coli strains resistant to Erythromycin 32mg/l

E. coli isolates resistant to 32 mg/l erythromycin were streak-plated on to blood agar containing doubling concentrations of erythromycin from 32 mg/l to 512 mg/l.

(iv) Genetic analysis of cefoxitin resistant strains

Bacterial Strains

B. fragilis strains B1, B265, B266, B269 and B271 were the parent strain and laboratory induced mutants isolated in the laboratories of LJV Piddock and R Wise (Dudley Road Hospital, Birmingham, England). B. fragilis NCTC 9343 (designated B108) was originally obtained from the National Collection of Type Culture (Colindale Road, London). B. fragilis B165 and B. thetaiotaomicron B171 were originally supplied by Dr B. Olsson-Liljequist (The National Bacteriology Laboratory, Stockholm, Sweden) while B. fragilis B264 and B. ovatus B263 were supplied by Dr Francis Tally (Tufts University School of Medicine). All strains (Table 5.3) were obtained directly from Dr Laura Piddock.

TABLE 5.3

Strain	Agar dilution MIC (mg/l) 10^4 cfu	Concentration of cefoxitin (mg/l) necessary to inhibit PBP by 50% (I50)[6] PBP 1	PBP 2	PBP 3	PBP 4
B. fragilis B1[2]	4	2	16	32	2
B. fragilis B265[3]	16	16	128	64	<1
B. fragilis B266[3]	16	1	32	32	<1
B. fragilis B269[3]	16	8	>256	64	32
B. fragilis B271[3]	32	2	64	32	256
B. fragilis B165[4]	32	8	4	32	4
B. fragilis B264[5]	64	8	8	2	16
B. thetaiotaomicron B171[4]	16	8	64	64	256
B. ovatus B263[5]	64	16	2	128	256

1 Data of Piddock and Wise (1987)
2 Parent strain identical to NCTC 9343 (B108)
3 Laboratory induced mutants
4 Strains supplied by Dr B. Olsson-Liljequist
5 Strains supplied by Dr F. Tally
6 Value derived from visual examination of fluorograph

Plasmid Isolation and Detection

Plasmids were isolated and detected by the modified method of Tinnell and Macrina (1976) as outlined in Chapter 2.

Preparation of Polyacrylamide Gels

SDS polyacrylamide (10%) slab gels were made with the buffer system of Laemmli (1970) as described by Poxton and Brown (1979). Table 5.4 shows gel recipe.

EDTA Extraction of Outer Membrane Proteins

Broth culture (50 ml) of each test strain was grown as previously described. Cells were harvested and washed twice in PAGE cell washing buffer [(pH 7.4) Appendix 4] by centrifugation at 10,000 g for 10 min at 4°C. The pellet was resuspended in 2 ml of PAGE EDTA buffer (Appendix 4) and placed in a 45°C waterbath for 30 min. The cells were placed in an ultrasonic bath (Dawe, Sonicleaner) for 10 min. The pellet was discarded and the preparation stored at -20° until required.

Sarkosyl Extraction of Outer Membrane Proteins

Broth culture (50 ml) of each strain was grown in PPY as previously described. Cells were harvested and washed once in 50 ml PBS (pH 7.2) by centrifugation at 10,000 g, for 10 min at 4°C. The cells were suspended in 5 ml distilled water and disrupted by sonication (using a Soniprep 150) at an amplitude of 6 - 10 u. Six one-minute bursts with a 30 second rest in between were sufficient to give more than 90% breakage. One volume of Sarkosyl was added to 9 volumes of broken cells and unbroken cells removed by centrifugation

TABLE 5.4 Preparation of Polyacrylamide Gels

Reagent	Volume (ml) to make separating gel (10%)*	stacking gel (4%)
Distilled water	6.95	3.5
Double-strength separating buffer	17.5	-
Double-strength stacking buffer	-	5.0
Acrylamide stock solution (40%)	8.75	1.0
TEMED	50 ul	20 ul
Ammonium persulphate (15 mg/ml)	1.75	0.5

* Percentage acrylamide in gel

at 10,000 g for 10 minutes at 4°C. The supernatant was centrifuged at 50,000 g for 1 h at 4°C to pellet insoluble outer membrane and washed once in 5 ml distilled water using a syringe and 26 g needle. Storage was at -20°C until required.

Protein Estimation (Folin)

Distilled water (350 ul) was added to protein sample (50 ul) to give a final volume of 0.4 ml. Sodium carbonate [12.5% (1.2 ml)] and copper sulphate [$CuSO_4.5H_2O$ (0.1 ml)] were added and the mixture was allowed to stand for 1 h at room temperature. Folin reagent was diluted 1 in 3 with distilled water and 0.2 ml was added to the mixture in each test tube. The contents of each tube were mixed and left to stand at room temperature for 25 min. Protein concentration was read at 750 mu against a reagent blank of distilled water taken through the assay.

Loading of Samples and Gel Electrophoresis

Protein solution (20 - 25 ug protein in 50 ul) was mixed with an equal volume of double-strength sample buffer (Appendix 4) and the protein washed once in 5 ml distilled water by similar centrifugation. The protein was denatured by heating for 3 min in a boiling water-bath. Samples (100 ul) were loaded into each cell. Electrophoresis was at 50 V (ca 25 mA) until samples had entered the separating gel (ca 1.5 h) and then at 150 V until samples had run ca 11 cm (approximately 3 h). The gel was then removed from the cassette and stained.

Staining Procedures for Proteins in Polyacrylamide Gels

Two staining methods were employed:

(i) Coomassie blue staining

Coomassie blue staining was by the method of Poxton and Sutherland (1976) using the solutions outlined in Appendix 4. The gel was fixed by overnight soaking in solution 1 and stained by immersion in solutions 2 - 5 for 1 h each with shaking.

(ii) Silver staining

Silver staining was by the modified method of Oakley (1980). The gel was fixed by overnight soaking in an aqueous solution of ethanol (25% v/v) and acetic acid (10% v/v) and then soaked in unbuffered glutaraldehyde (10% v/v) for 30 min. It was rinsed in 500 ml water for 10 min. The rinsing step was repeated and the gel soaked in 1,000 ml distilled water for 2 h followed by soaking in dithiothreitol (5 mg/ml) for 30 min.

The gel was drained of dithiothreitol solution and freshly prepared ammoniacal silver nitrate solution [1.4 ml ammonia solution (SG 0.88) was added to 21 ml 0.36% NaOH. Silver nitrate solution (4 ml of a 19.4% solution) was added slowly with vigorous agitation and the volume of liquid made up to 100 ml.] The gel was stained for 15 min, removed from the silver solution and washed in four changes of distilled water (200 ml) over 40 min. It was transferred to fresh citric acid (0.005%) in formaldehyde (0.019%) and allowed to develop for 10 min then washed four times in 1,000 ml distilled water. The whole staining procedure, with the exception of fixing, was conducted on a shaker.

RESULTS

Curing Experiments

The subinhibitory concentration of ethidium bromide

The number of organisms per ml of overnight culture of V479 was found to vary between 10^8 and 10^9. The results obtained for the seven determinations were 8.6×10^8, 1.51×10^9, 1.34×10^9, 2.20×10^9, 1.7×10^9, 2.4×10^9 and 2.97×10^9 cells per ml. The average value was 1.85×10^9. In all subsequent curing experiments overnight cultures were diluted by a factor of 10^4.

After 24 h incubation in the presence of ethidium bromide growth was visible at concentrations of 8 and 16 mg/l and after 48 h growth was additionally visible at concentrations of 20 and 24 mg/l. At an ethidium bromide concentration of 20 mg/l there was a marked reduction in growth but the growth was just barely visible at 24 ug/ml. There was no growth in the presence of ethidium bromide 32 ug/ml. The subinhibitory concentration of ethidium bromide was taken as 20 mg/l and this concentration used for all curing experiments with the test strains.

The effect of ethidium bromide on curing of resistance

Clindamycin and erythromycin resistance were not cured in any of the strains tested.

The Transfer of Clindamycin and Erythromycin Resistance

Bacteroides to *Bacteroides* transfer was observed from *B. fragilis* V479 to *B. fragilis* MH47 (Table 5.5). The transfer frequency, calculated as the number of transcipients per donor cell per ml of mating mixture, was 2.5×10^{-7}. The plasmid profile of the donor, recipient and transcipient strains are shown in Fig 5.3. The plasmid content of the donor (V479) and transcipient strains were identical. The recipient strain (MH47) was a plasmid-free strain. Resistance to clindamycin and erythromycin were transferred together as transcipient strains acquired resistance to both of these antibiotics. Transfer was not observed in any other mating experiment.

The *E. coli* strains had MICs to erythromycin of 32 mg/l. During mating experiments with MH2 and MH12 it was possible to isolate mutants with MICs of 64 mg/l. However after mating experiments with *B. fragilis* MH11 it was possible to isolate mutants with resistance to 512 mg/l of erythromycin. These mutants arose at low frequency, approximately 1.1×10^{-6} and high level resistance was stable through several sub-cultures.

DNA Hybridization Studies

DNA hybridization was not observed between the plasmid and chromosomal DNA of the test strains and the resistance determinant carried by *E. coli* V1476.

TABLE 5.5 Mating Pairs and Transfer Frequencies for *Bacteroides* to *Bacteroides* Antibiotic resistance Transfer

Mating Experiment	Donor Strains	Recipient Strains	Antibiotics used in Selection[1]	Transfer Frequency[2]
1	*B. fragilis* V479	*B. uniformis* MH43	PEN Cc	-[3]
		B. fragilis MH47	CTX Cc	2.5×10^{-7}
2	*B. fragilis* MH2	*B. fragilis* MH43	PEN Cc	-
		B. fragilis MH47	CTX Cc	-
3	*B. fragilis* MH11	*B. uniformis* MH43	PEN Erm	-
		B. fragilis MH47	CTX Erm	-
4	*B. fragilis* MH12	*B. uniformis* MH43	PEN Cc	-
		B. fragilis MH47	CTX Cc	-

[1] Cc - clindamycin CTX - cefotaxime PEN - penicillin

[2] Number of transcipients per donor cell per ml of mating mixture

[3] - No transfer

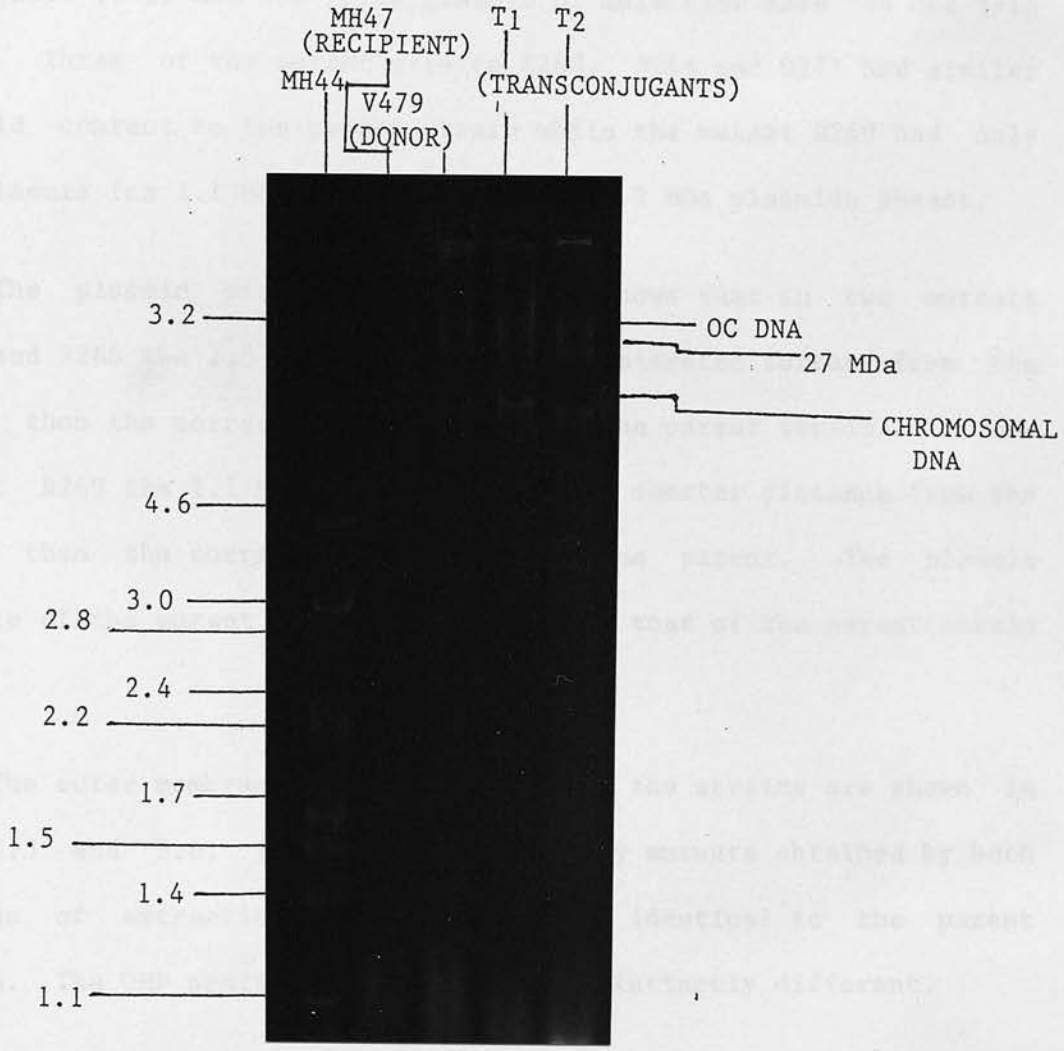


Figure 5.3 Agarose gel (0.6%) showing the plasmid profiles of strains from the mating of a clindamycin resistant (Ccr) donor V479 and a clindamycin sensitive recipient MH47 to produce Ccr transconjugant strains T_1 and T_2 with identical plasmid profiles to the donor.

Genetic Analysis of Cefoxitin Resistant Strains

Plasmids were not detected in any of the naturally resistant strains. The cefoxitin sensitive parent strain B. fragilis B1 had three plasmids of molecular mass 2.9, 1.5 and 1.1 MDa while strain B108 (NCTC 9343) had one large plasmid of molecular mass 44 MDa (Fig 5.4). Three of the mutant strains B265, B266 and B271 had similar plasmid content to the parent strain while the mutant B269 had only one plasmid (ca 1.1 MDa) with the 1.5 and 2.9 MDa plasmids absent.

The plasmid profile of the strains shows that in two mutants B265 and B266 the 1.5 and 1.1 MDa plasmids migrated further from the wells than the corresponding plasmids in the parent strain. In the mutant B269 the 1.1 MDa plasmid migrated a shorter distance from the wells than the corresponding plasmid in the parent. The plasmid profile of the mutant B271 was identical to that of the parent strain B1.

The outer membrane protein profiles of the strains are shown in Figs 5.5 and 5.6. Those of the laboratory mutants obtained by both methods of extraction and straining were identical to the parent strain. The OMP profile of NCTC 9343 was distinctly different.

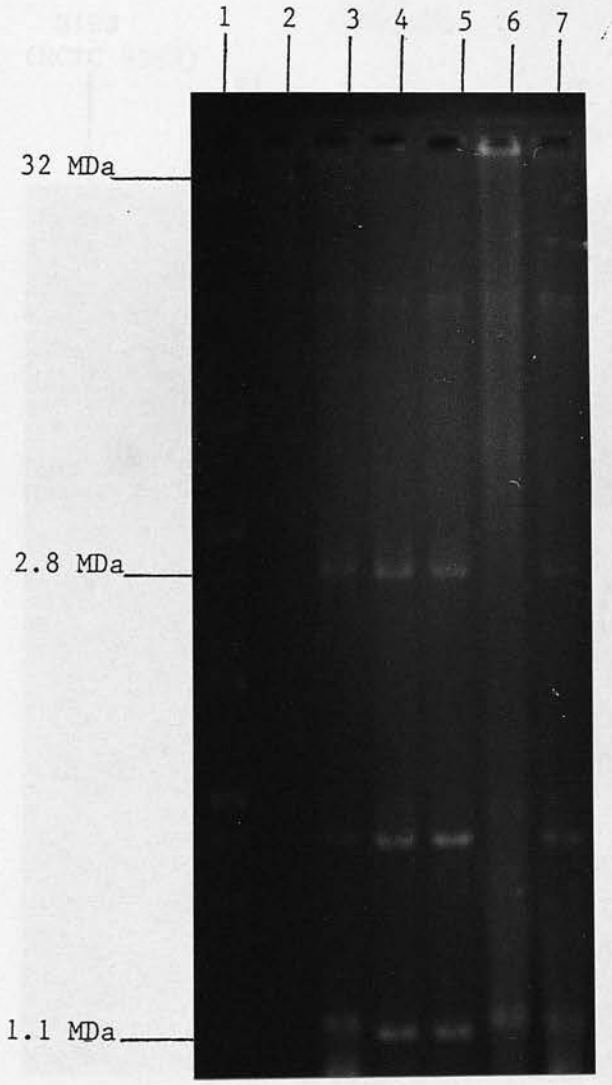


Figure 5.4

Agarose gel (0.6%) showing plasmid profiles of
(1) standard strain MH44; (2) B108 (NCTC 9343) (4) cefoxitin; sensitive parent strain, B1; (4) mutant strain B266
(5) mutant strain B265 (6) mutant strain B269 (7) mutant strain B271.

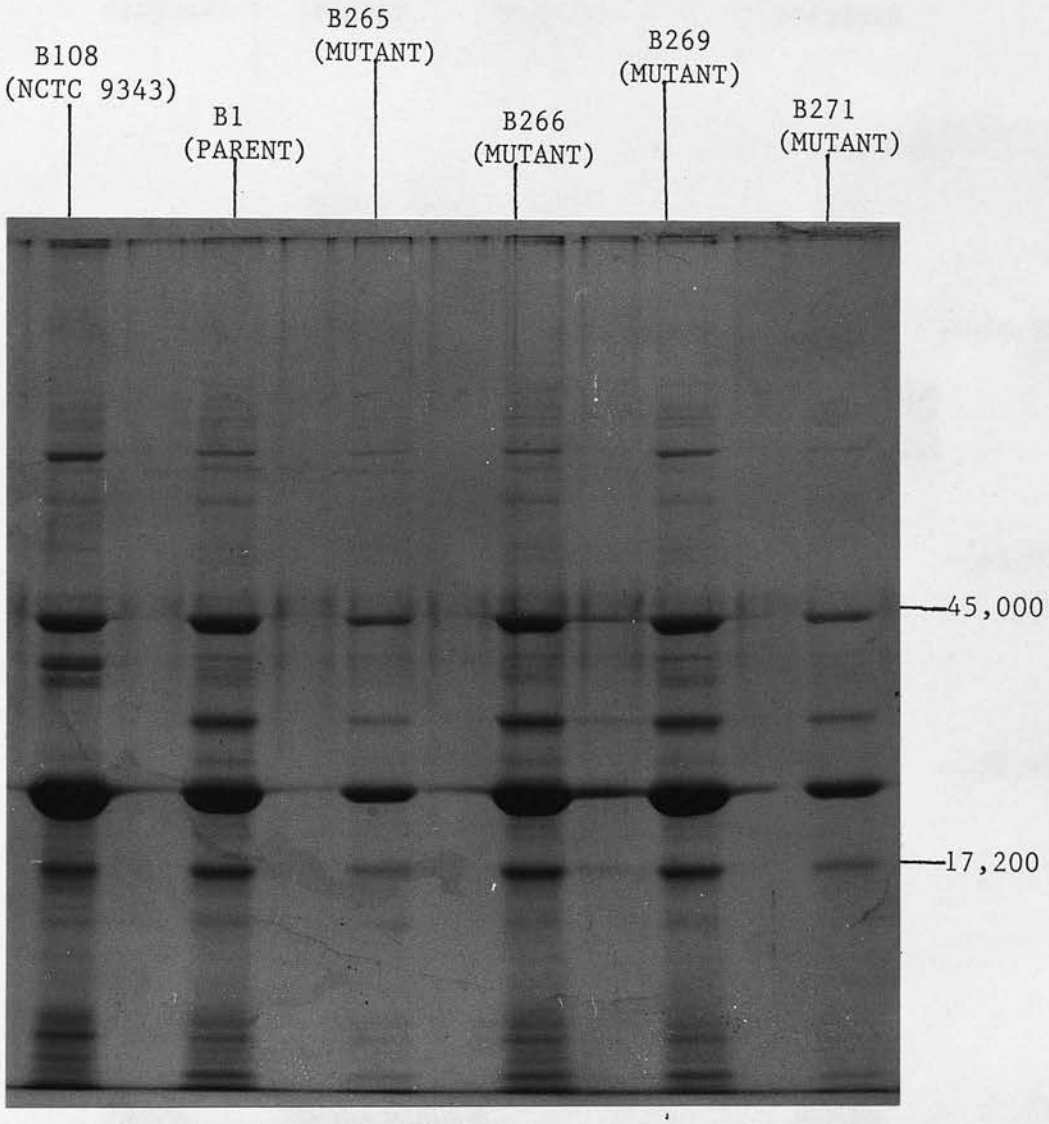


Figure 5.5

Coomassie blue stained SDS-PAGE of sarkosyl extracts of outer membrane proteins of B. fragilis strains B108 (NCTC 9343 reference strain) B1 (cefoxitin sensitive parent strain) B265, B266, B269, B271 (cefoxitin mutants of B1).

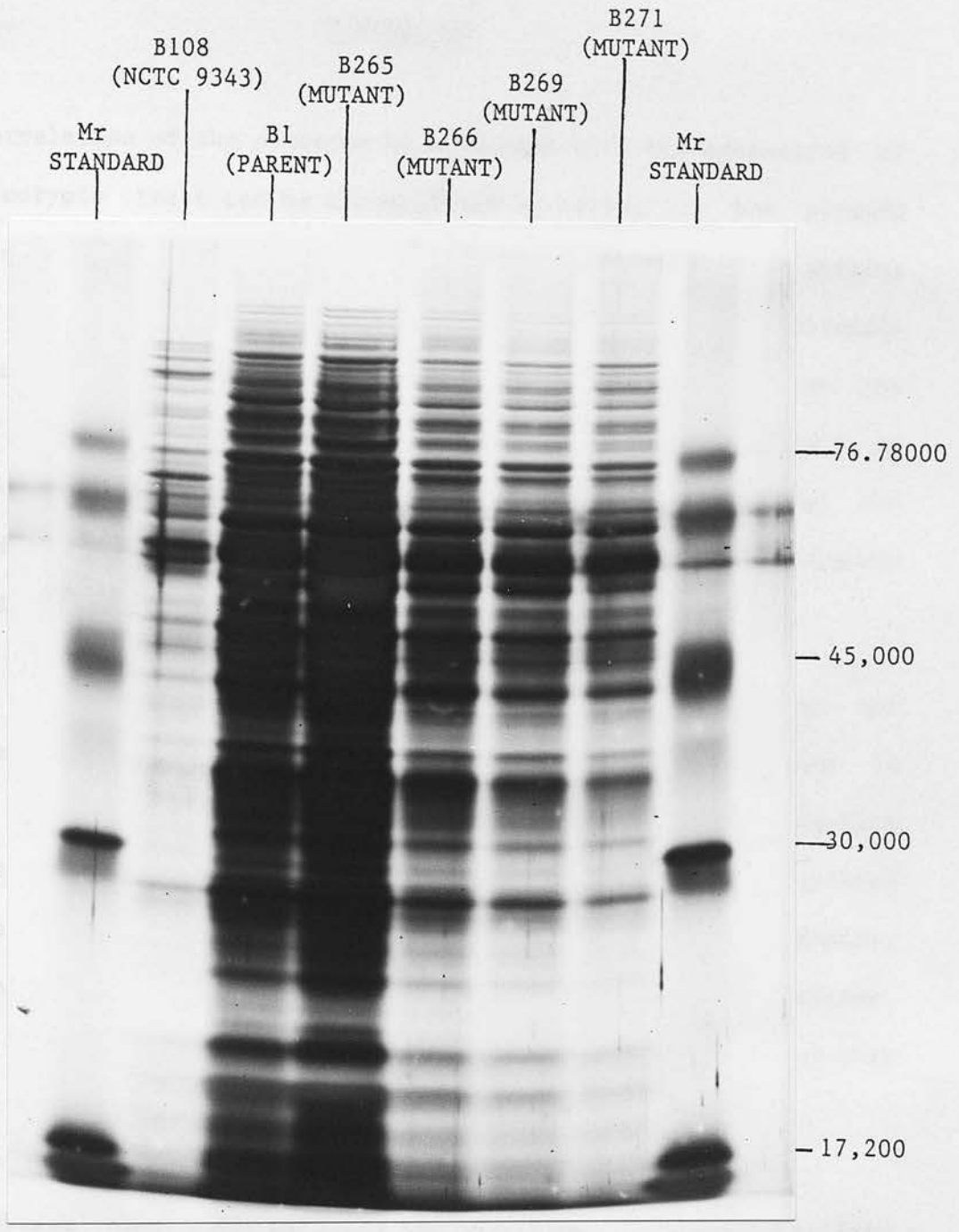


Figure 5.6

Silver stained SDS-PAGE of EDTA extracts of the outer membrane proteins of B. fragilis strains B108 (NCTC 9343, reference strain), B1 (cefoxitin sensitive parent strain) and B265, B266, B269, B271 (cefoxitin resistant mutants of B1) and of the molecular mass (Mr) standard.

DISCUSSION

Correlation of the presence of a plasmid with the expression of a phenotypic trait can be accomplished by curing of the plasmid involved or the transfer of its genetic determinants into strains lacking the characteristic under investigation. In cases of plasmid-mediated antibiotic resistance, sensitive strains may arise from the loss of plasmids due to errors in replication and segregation and to deletions. There is variation in the loss of plasmid DNA but the rate of spontaneous loss can be increased by exposure to various physical and chemical agents which are known as curing agents.

Curing agents include acriflavine, acridine orange and ethidium bromide but eithidium bromide was chosen for this study as it was shown by Rotimi and Duerden (1982) to be the most effective with Bacteroides species. While some strains which displayed resistance to clindamycin and erythromycin did not have plasmids, resistance was not cured in any of the eleven resistant strains. Failure to cure a phenotypic trait is not however absolute proof that the trait is chromosomally mediated.

Problems have resulted from the isolation of uncharacterised plasmids due to current investigation. There is no standard curing agent and curing agents are individually effective only against some plasmids (Grinstead et al., 1984). The plasmids involved may therefore be refractory to ethidium bromide. In addition the plasmid may also encode functions vital to the viability of the cell, resulting in the death of cured cells.

Clindamycin-erythromycin resistance in B. fragilis V479 is known to be encoded by a transposon Tn4351 located on a 27 MDa plasmid designated pBF4 (Welch et al., 1979; Shoemaker et al., 1985). Attempts to transfer this plasmid into the recipient strain B. uniformis MH43 were unsuccessful. Resistance was however transferred to B. fragilis MH47. This transfer confirmed the fact that failure in other mating experiments was not due to the transfer technique. Welch et al. (1979) postulated that there may be interstrain differences in surface receptors required for plasmid transmission or differences in the ability of plasmids to replicate or be expressed in a given host strain. Alternatively, lack of resistance transfer in strains with uncharacterised plasmids could indicate that the genes conferring clindamycin and erythromycin resistance are chromosomally located or that the plasmids on which they occur lack mobilization factors.

The occurrence of high level resistance mutants following mating experiments between E. coli and Bacteroides has not been previously reported. Mutations to erythromycin in E. coli usually gives rise to strains which are resistant to one dilution above the MIC. However these strains were resistant to five times the normal MIC and resistance was stable. Plasmid DNA was not detected in these isolates and the genetic basis remains uncertain. This may indicate a role of Bacteroides in increasing resistance in facultative anaerobes when present together in concentrated culture. In the gut where the number of organisms is high the Bacteroides may exert this effect on E. coli. This observation may have therapeutic implications for mixed infections.

High level resistance in Bacteroides is usually accompanied by cross-resistance to erythromycin and the streptogramins (MLS resistance) and has been shown in several strains to be plasmid-mediated (Privatera et al., 1981; Tally et al., 1979; Welch et al., 1979). However only three of the ten strains showing high level resistance to clindamycin and/or erythromycin had plasmids. In a fourth strain MH79 plasmids could not be consistently isolated and the plasmid DNA involved was possibly unstable. These results indicate that high level resistance to clindamycin and erythromycin can be chromosomally encoded. Callihan et al. (1984) found that in some plasmid containing strains a resistant determinant which hybridizes with the determinant from pBF4 was carried on the chromosome. It is possible therefore that in the plasmid-containing strains MH2, MH12 and MH11 the resistant determinant carried on pBF4 resides on the chromosome.

Four self-transmissible plasmids conferring high level resistance to clindamycin and erythromycin have been described in the Bacteroides (Guiney et al., 1984; Smith and Macrina, 1984; Tally et al., 1979, 1982; Welch et al., 1979). These plasmids vary in size and restriction endonuclease patterns but DNA hybridization studies have shown the existence of homology between the MLS determinants (Guiney et al., 1984; Marsh et al., 1983; Shimell et al., 1982; Smith, 1985). This homology has resulted in the theory of widespread dissemination of an MLS resistance determinant in Bacteroides (Rasmussen et al., 1987). Investigations of low-level, chromosomally encoded resistance to clindamycin has shown an absence of homology indicating the existence of two different

determinants. The absence of homology between the determinant of the TN4351 transposon carried by E. coli V1476 and the plasmid or chromosomal DNA of the test strains indicate the existence of another resistance determinant encoding high level resistance to clindamycin and erythromycin.

It has been postulated that cefoxitin resistance to beta-lactamase is a major factor in its superior activity, compared to the cephalosporins, against the bacteroides (Darland and Birnbaum, 1977; Neu, 1983; Sweet et al., 1979; Tally et al., 1979; Weinrich and Del Bene, 1973). Several groups of investigators have demonstrated hydrolysis of cefoxitin by novel beta-lactamases produced by some cefoxitin-resistant B. fragilis strains (Cuchural et al., 1986a; Yotsuji et al., 1983). However, although the association between beta-lactamase susceptibility and antibacterial action is very significant statistically, the relationship is not absolute. Several B. fragilis isolates have been found to be resistant to cefoxitin though no beta-lactamase hydrolysis could be detected (Darland and Birnbaum, 1977; Olsson et al., 1980; Piddock and Wise, 1987). Darland and Birnbaum (1977) demonstrated the absence of a major permeability barrier but Dornbusch et al. (1980), in the absence of beta-lactamase activity, deduced that cefoxitin resistance was due to an intrinsic factor which was possibly alterations in the cell wall.

The observation that the OMP profile of the parent strain B. fragilis B1 was identical to that of the B. fragilis strain NCTC 9343 led Piddock and Wise (1987) to postulate a correlation between outer membrane proteins and cefoxitin resistance. However, in our hands the OMP profile of NCTC 9343 differed from that of B1 despite

its identical PBP profile to 34 beta-lactam antibiotics. This suggests that OMP profiles may be strain specific and differences observed by Piddock and Wise (1987) may bear no relationship to cefoxitin resistance.

Similarly, the plasmid profile of NCTC 9343 differed from that of B1 despite the identical MIC value to cefoxitin. It is possible that homology existed between the genetic material carried on the plasmid DNA of both strains but DNA hybridization studies were not conducted due to time restraints. The differences in plasmid profile of the parent and mutant strains indicate plasmid involvement in cefoxitin sensitivity.

A limitation of the method of agarose gel electrophoresis for the determination of the molecular mass of plasmid DNA is that small differences in plasmid size such as those observed in Fig 5.1 (strains B265 and B266) cannot be accurately determined using a logarithmic plot. In two mutants, strain B266, for which Piddock and Wise (1987) could find no mechanism of cefoxitin resistance, and strain B265, two plasmids (molecular mass 2.9 and 1.5 MDa) showed slight alterations in size which could be due to insertion or deletion of base pairs. In mutant strain B269 a four-fold increase in MIC to cefoxitin was accompanied by the loss of two plasmids (the 2.9 and 1.5 MDa plasmids) and an alteration of molecular mass of the third plasmid (the 1.1 MDa plasmid). The plasmid profile of B271 was identical to that of the parent strain B1 despite the eight-fold decrease in cefoxitin sensitivity.

Four mutant strains with distinct differences in plasmid DNA and PBP competition assay profiles were isolated from a single parent. This would indicate that the mutagen NTG did in fact produce multiple mutations of the parent strain. The results of this study have shown that these genetic mutations involved plasmid DNA. Crumplin and Smith (1981) provided evidence that R factors in facultative anaerobes may increase their susceptibility to antimicrobial agents. It is possible therefore that the observed alterations of plasmid DNA may have resulted in the decreased sensitivity to cefoxitin.

Piddock and Wise (1987) deduced that for three of the strains (B265, B269 and B271) decreased sensitivity to cefoxitin was due to alterations in PBP profile during binding assays with cefoxitin. The PBP profile of the mutant strains may be related to the plasmid profile. An examination of the PBP profile of the strains (Table 5.1) shows that of all four mutants, B269, the strain with two missing plasmids, had the greatest alteration in PBP profile relative to the parent strain. In B271 where no changes in plasmid profile were observed the effect of the mutagen on the plasmid DNA may have been a functional rather than a structural one. However, the absence of plasmids in naturally occurring cefoxitin-resistant strains may have indicated that in those strains resistance was chromosomally mediated. It is possible that the four to eight-fold increase in the MIC to cefoxitin of mutant strains of B. fragilis B1 may also be due to alterations in chromosomal DNA. This is contrary to the observations of Raschtchian et al. (1982) who deduced that cefoxitin-resistance genes may be carried on plasmid DNA or on an episome.

CHAPTER 6

PLASMID DNA AND CAPSULE VARIATION IN *BACTEROIDES FRAGILIS*

INTRODUCTION

The observation that Bacteroides fragilis, a minor component of the faecal flora, is more frequently associated with infection than other members of the Bacteroides fragilis group, implies the existence of unique virulence factors associated with its pathogenicity. Studies with the capsule of this species, a putative virulence factor, have shown that there is variation in capsule size (Babb and Cummins, 1978; Patrick et al., 1986). Capsules ranging from a half to four times the cell diameter have been described by Babb and Cummins (1978).

Two extracellular layers, a narrow electron dense layer and a fibrous metwork, have been observed by electron microscopy (Lambe et al., 1984; Patrick et al., 1986). It was suggested that these structures were identical, with differences in appearance due to condensation during preparations for viewing (Lambe et al., 1984). However, Patrick et al. (1986) showed that they may, in fact, be different in nature. This group separated bacteria with different capsule sizes from a single population by the use of four step (20, 40, 60, 80%) Percoll density gradients (Table 6.1, Figs. 6.1 - 6.4).

Patrick (1986) extended this work to determine the ability of cells from the two extreme interfaces (0 - 20% and 60 - 80%) to agglutinate erythrocytes (Table 6.2). She showed that cells from the 60 - 80% interface gave strong haemagglutination with both the rocked tile and static settling tests. No agglutination was observed with cells from the 0 - 20% interface.

TABLE 6.1 Incidence of fibrous network and electron dense layer in *Bacteroides fragilis* (NCTC 9343) after density gradient separations*

Structure	Gradient position			
	0 - 20% interface	20 - 40% interface	40 - 60% interface	60 - 80% interface
Fibrous network	++	+	+	-
Electron dense layer	+	+	+	+
Figure	6.1	6.2	6.3	6.4

++ Extensive fibrous network

\+ Presence of structure

\- Absence of structure

* Results of Patrick *et al.* (1986)

Figures 6.1 - 6.4

Electron micrographs of B. fragilis 9343 to show surface structures of cells harvested from the interface layers of a four-step Percoll density gradient. (courtesy of S. Patrick)

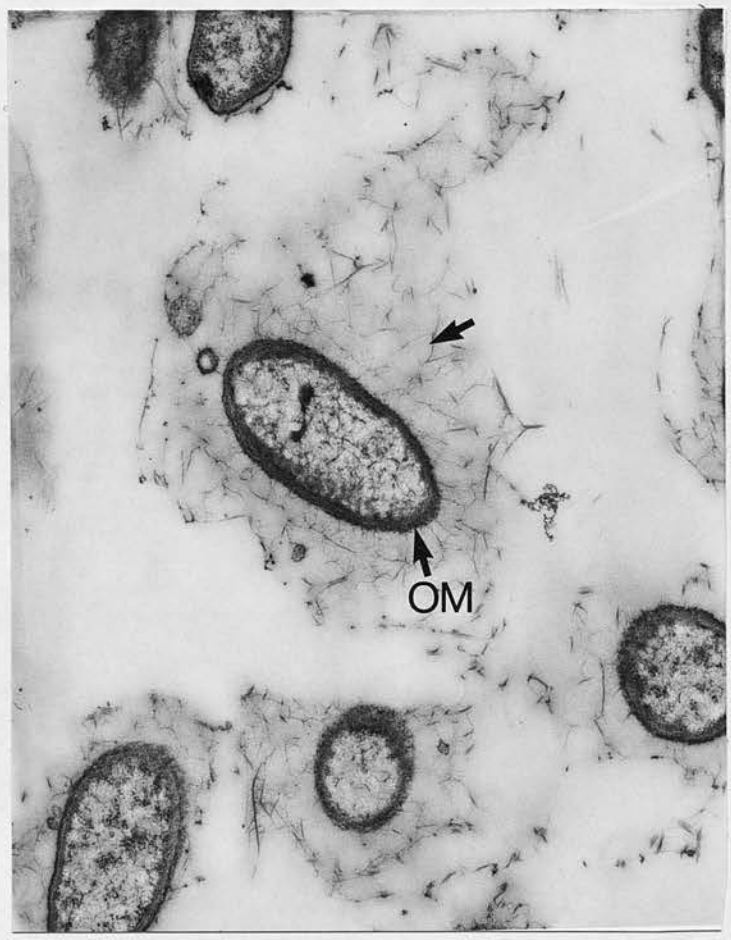
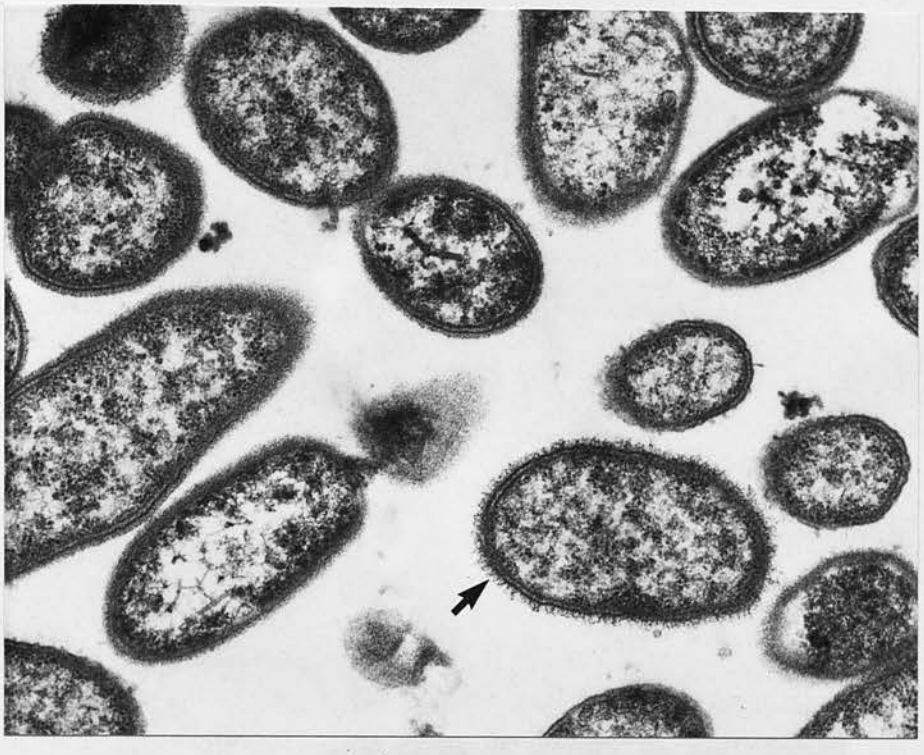
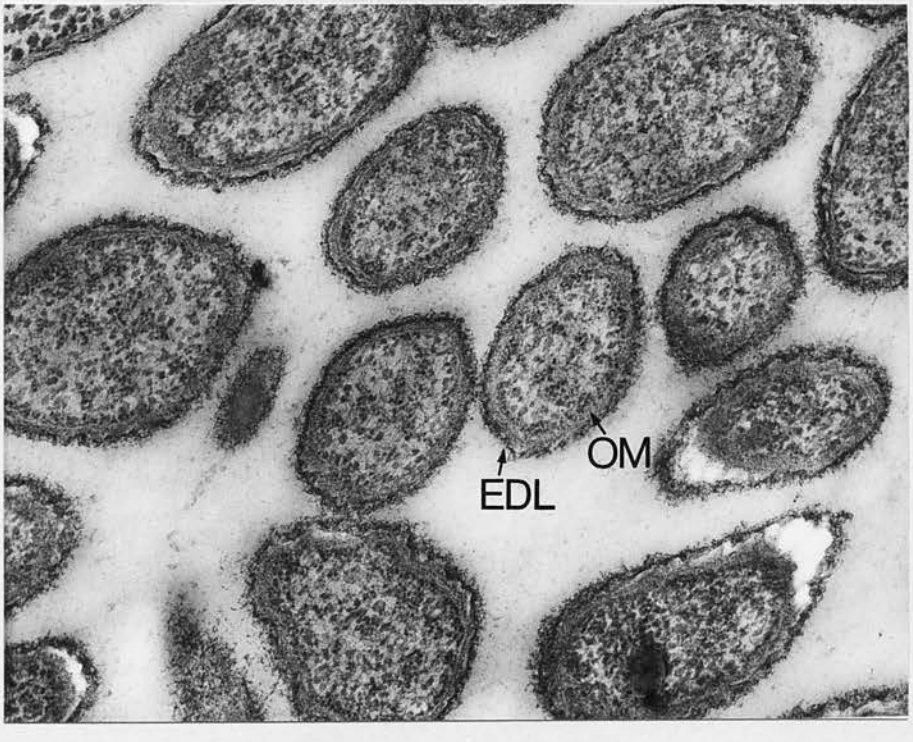


Figure 6.1

Cells from the 0 - 20% interface showing the fibrous network (arrowed) surrounding the outer membrane (OM).



(6.2)



(6.3)

Figures 6.2 and 6.3

Cells from the 20 - 40% interface (6.2) and the 40 - 60% interface (6.3) showing a narrow electron dense layer (EDL) adjacent to the outer membrane (OM) or a small fibrous network (arrowed).

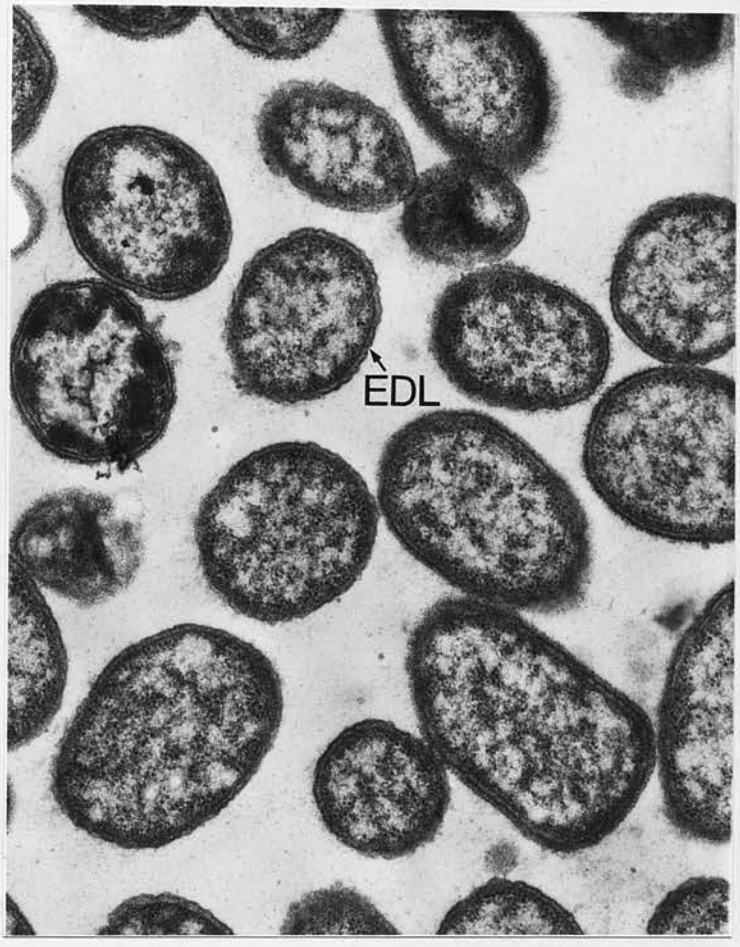


Figure 6.4

Cells from the 60 - 80% interface showing the absence of a fibrous network but the presence of the electron dense layer (EDL).

TABLE 6.2 Comparison of haemagglutination of a number of erythrocyte species by B. fragilis subcultured from the 0 - 20% and 60 - 80% interface layers of a step density gradient*

Strain	Interface layer	Erythrocyte species								
		Human			Horse	Guinea pig	Rabbit	Sheep	Chicken	Mouse
		O	A	B						
NCTC 9343	0 - 20%	-	-	-	-	-	-	-	-	-
	60 - 80%	+	+	+	+	+	+	+	+	+
NCTC 9344	0 - 20%	-	-	-	-	-	-	-	-	-
	60 - 80%	+	+	+	+	+	+	+	+	+
GNAB4	0 - 20%	-	-	-	-	-	-	-	-	-
	60 - 80%	+	+	+	+	+	+	+	+	+

+ = haemagglutination within 5 minutes

- = no haemagglutination

* Data of Patrick (1986)

The proportion of encapsulated cells and the stability of segregation are also subject to variation (Babb and Cummins, 1978; Burt et al., 1978; Patrick et al., 1986). Babb and Cummins (1978) showed that the chemical, serological and possibly biological properties of capsule-producing strains, may be altered by growth conditions. No work has been done on the genetic basis of capsule production. A study of three of the strains was conducted to extend the work of Patrick (1986) and Patrick et al. (1986), and to determine the possible involvement of plasmid DNA in capsule variation.

METHODS

Bacterial Strains

Three of the six strains examined by Patrick (1986) were investigated. *B. fragilis* strains 9343 and 9344 were obtained from the National Collection of Type Cultures (NCTC). *B. fragilis* strain GNAB4 was obtained from the culture collection of the Microbial Pathogenicity Research Laboratory of the Bacteriology Department, University of Edinburgh.

Bacterial Culture Methods

Bacteria were grown to late log phase in PPY broth as previously described (Chapter 2).

Separation of Bacteria

Bacteria were separated on Percoll (polyvinylpyrrolidone (PVP-) coated colloidal silica) gradients by the modified method of Kurnick *et al*. (1979). Two step discontinuous gradients were generated in 4 ml sterile culture tubes (12 mm x 75 mm). The tubes were wetted with a small amount of new born calf serum (NBCS) to allow even flow of the Percoll. A stock solution of Percoll isotonic with physiological saline (PBS) was prepared by diluting nine volumes of Percoll with one volume of ten-fold PBS (pH 7.2). This stock was referred to as 100% Percoll. Less dense solutions of Percoll were prepared by the addition of suitable volumes of the growth medium (PPY).

Percoll (2 ml of 20% or 80%) was added to each tube. Cells from 5 ml of broth culture were pelleted by centrifugation in a bench-top centrifuge for 20 min at 2600 g. The cells were resuspended in 5 ml pre-reduced PPY and 2 ml was layered on to the Percoll. The gradients were centrifuged for 20 min at 2,500 g. Cells were harvested from the 0 - 20% interface and from the bottom of the tubes containing 80% Percoll. Harvesting was from above by means of a Pasteur pipette.

Unwashed cells from the gradient were used as a direct inoculum for 10 ml of pre-reduced PPY. This was grown to late log phase to produce cultures enriched for cells harvested from the gradient. Cells from the enriched cultures were pelleted, resuspended in PPY and the separation procedure repeated two to three times. Finally cells harvested from the gradient were used as the inoculum of broth cultures used for plasmid analysis.

Preparation of Plasmid DNA

Broth cultures (50 ml) were grown and plasmid DNA isolated and visualised as previously described (Chapter 3).

Observation of Capsules

The presence and sizes of capsules was determined by light microscopy with India ink and basic fuchsin (Cruickshank et al., 1965). Observations were made of cultures before and after enrichment.

It was impossible to obtain cultures of cells with uniform capsule size despite repeated separation and enrichment, (Fig 6.5 and 6.6). Prior to enrichment the proportion of cells with capsules was <1% for B. fragilis NCTA 9343, <10% for B. fragilis NCTC 9344 and between 10 and 20% for B. fragilis GNAB 4. This was increased to approximately 10% for B. fragilis NCTC 9343, 25% for B. fragilis NCTC 9344 and 50% for B. fragilis GNAB 4 by repeated enrichment. The effect of enrichment on cells which produced an electron dense layer* was less marked. During separation, before and after enrichment, the majority of cells collected at the 0 - 80% Percoll interface, with only a small proportion of cells (increased slightly by enrichment) sedimenting to the bottom of the 60% Percoll layer.

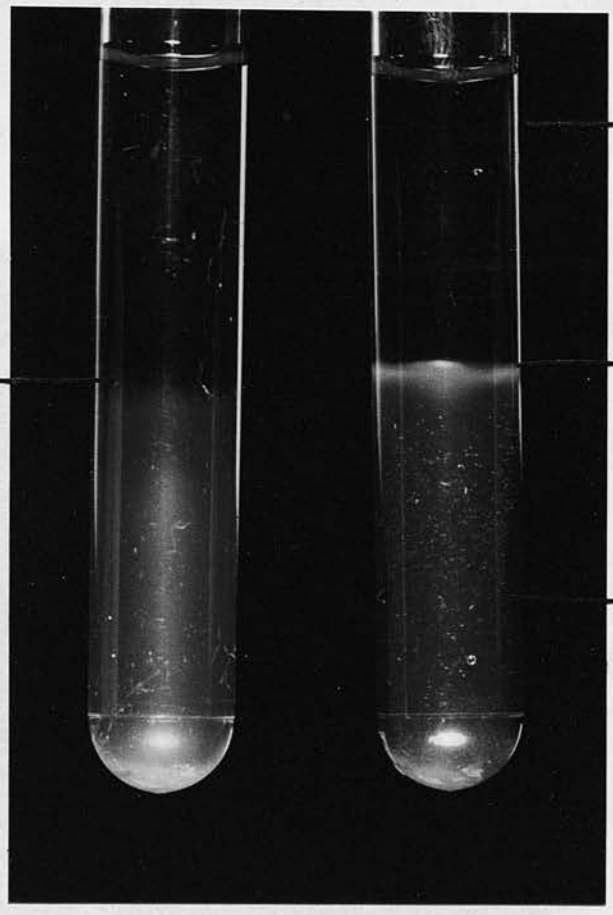
The plasmid profile of cells enriched for large capsules and electron dense layers* were identical (Fig 6.7). A large plasmid of molecular mass 44 MDa was isolated from NCTC 9343 but plasmids were not detected in strains NCTC 9344 and GNAB4.

*as claimed by Patrick et al.(1986).

Figure 6.5

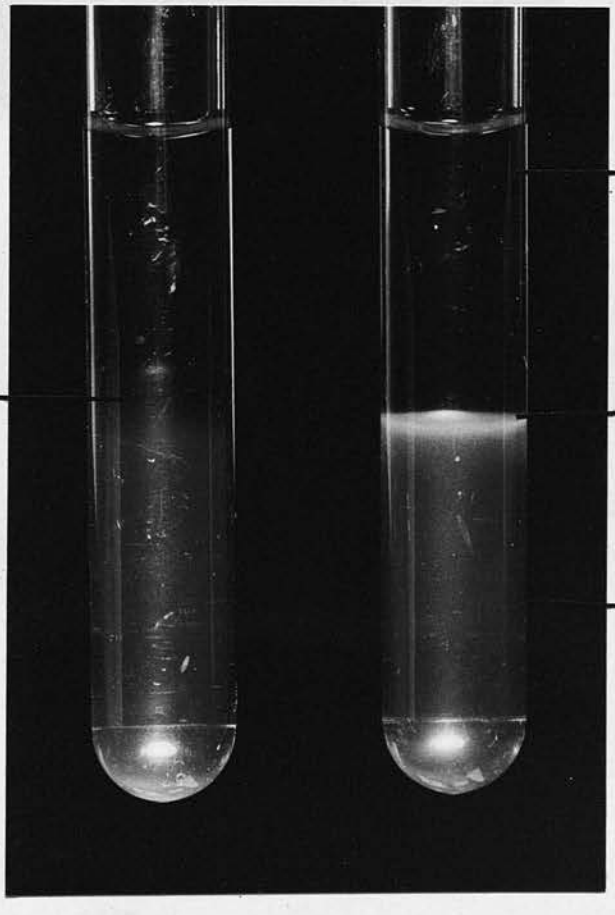
Enrichment on Percoll gradients of capsulate cells of three strains of bacteroides

a b

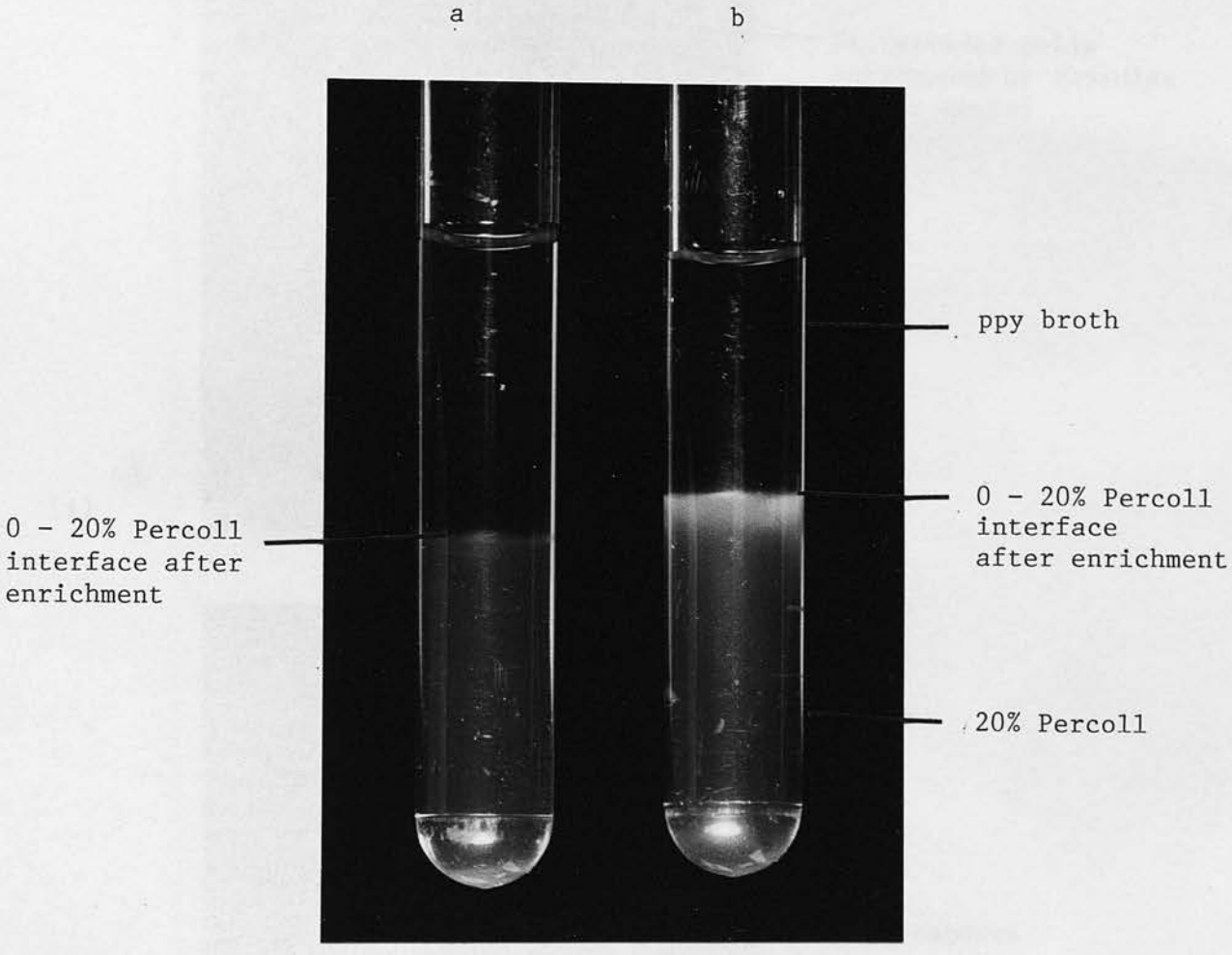


(i) NCTC 9343

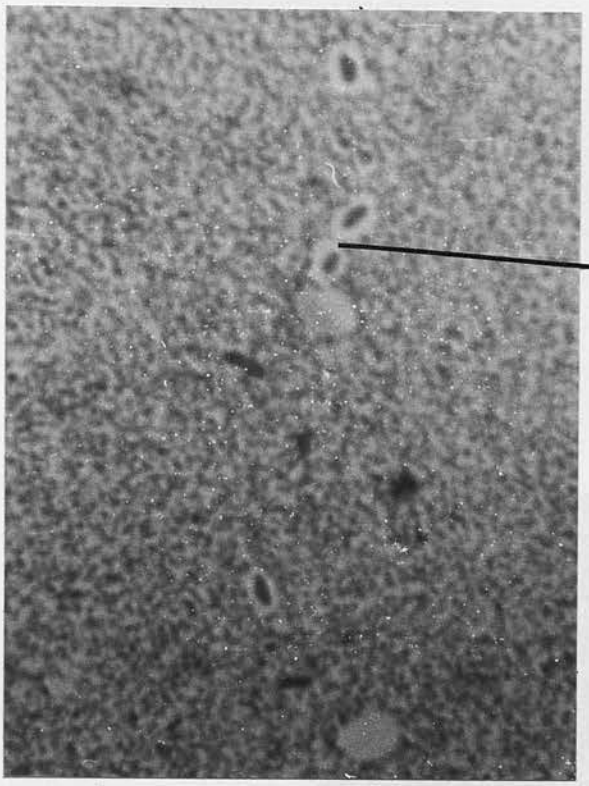
a b



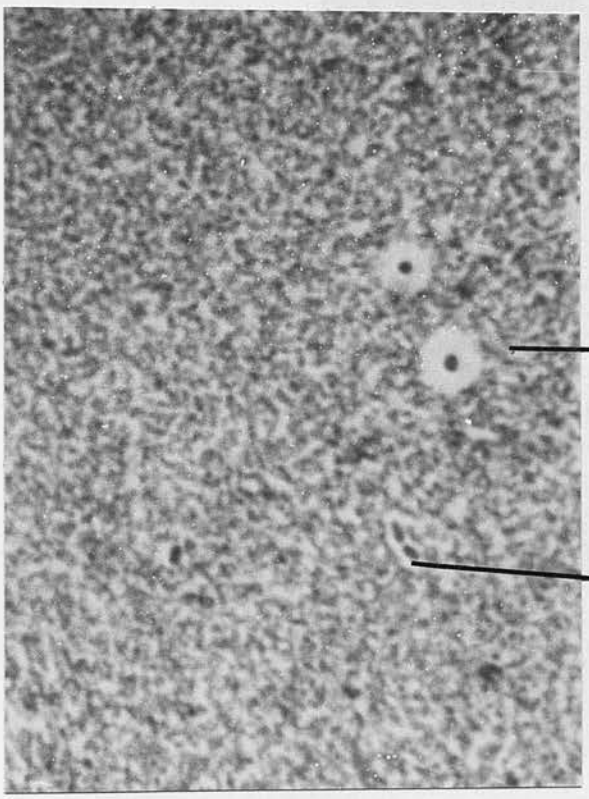
(ii) NCTC 9344


a
b
ppy broth
0 - 20% Percoll interface after enrichment
0 - 20% Percoll interface after enrichment
20% Percoll

(iii) GNAB 4



(i)



(ii)

Figure 6.6

Encapsulated cells of Bacteroides fragilis as visualised in India ink films under light microscopy (magnification 4300x)

(i) Cells of NCTC 9343 (ii) Cells of NCTC 9344

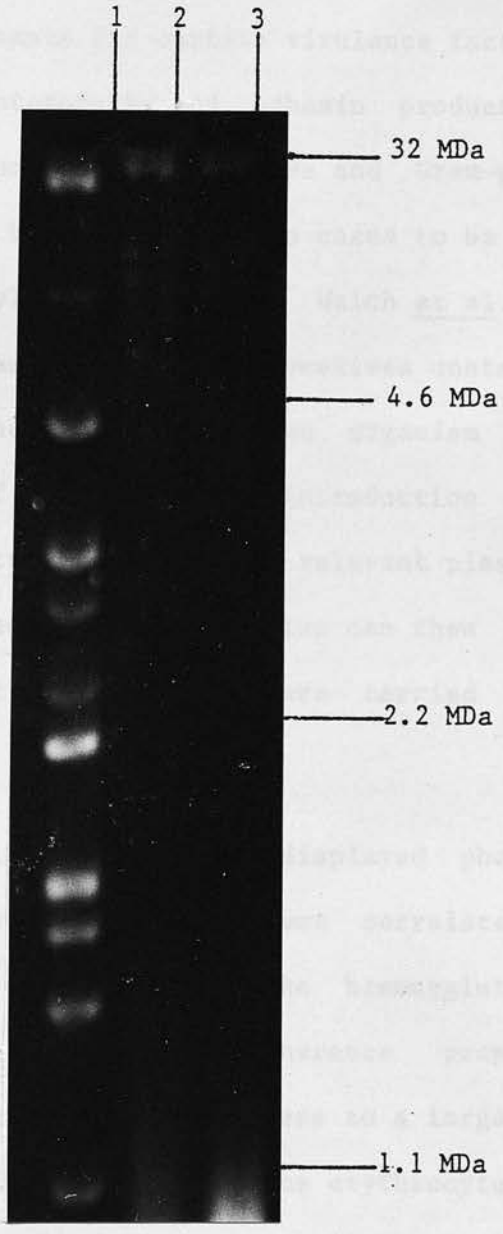


Figure 6.7

Agarose gel (0.6%) showing plasmids isolated from (1) the standard strain B. Fragilis MH44, (2) cells of B. Fragilis NCTC 9343 harvested from the 0-20% interface of a Percoll gradient and enriched for fibrous capsule, and (3) cells of B. Fragilis NCTC 9343 harvested from the bottom of a two-step Percoll gradient (containing 0% and 80% Percoll) enriched for an electron dense layer (EDL).

DISCUSSION

Adherence of pathogenic bacteria to different kinds of substances is important in successful colonisation of the host tissue. Mechanisms of adherence therefore constitute virulence factors. The genetic determinants for certain virulence factors can be carried on plasmids. Enterotoxin and adhesin production in E. coli and antibiotic resistance in Gram-negative and Gram-positive bacteria, for example, have been shown in some cases to be plasmid mediated (Helinski, 1973; Gyles et al., 1974; Welch et al., 1979; Elwell and Shipley, 1980). These plasmids are sometimes unstable and in such cases changes in the virulence of an organism can be correlated with the loss of plasmids. Re-introduction of the plasmids into the wild-type strains lacking the relevant plasmid or transfer of the plasmid to other recipient strains can then confirm that genes coding for a particular character are carried on the plasmid.

The bacteroides subpopulations examined, displayed phenotypic variation in haemagglutinating activity which was correlated with differences in extracellular structures.* The haemagglutinating activity of bacteria is related to its adherence properties. Adhesins can confer on cells the ability to adhere to a large number of cellular substrates within the host, including erythrocytes (Old, 1985). It was considered possible that this variation could be due to the presence of extrachromosomal DNA which was being transferred through a population of cells. The identical nature of the plasmid profile of each strain when cells enriched for large capsules were

* by Patrick (1986)

compared with those enriched for electron dense layers indicated that differences were due to gene expression rather than the presence or absence of plasmid DNA. Further evidence for this was provided by the fact that in two of the strains in which capsule variation was observed no plasmids were present.

Despite repeated enrichment attempts it was impossible to obtain a culture with uniform capsule size. This was in agreement with the findings of Patrick et al. (1986). These results indicate that incomplete segregation of capsules did not result from the transfer of plasmid DNA within a population of cells. However, the inability to produce pure cultures of cells with the fibrous capsule may have altered the plasmid profile of the strains. Low copy number plasmids present in these strains could have remained undetected. Enrichment for cells with an electron dense layer in the absence of a fibrous network, was from an inoculum harvested from the bottom of a 0 - 80% Percoll gradient. Despite the fact that Patrick et al. (1986) harvested cells with the EDL from the 60 - 80% interface of a Percoll gradient, this modified method has been shown to give good results (S. Patrick, personal communication).

The presence of several capsule sizes within a given population of cells may increase the virulence of strains. Bacteroides cells producing the fibrous capsule, when present in concentrations greater than 10^7/ml, have been shown to inhibit phagocytic and serum killing of both capsule producing cells and concomitant facultative anaerobes (Connolly et al., 1984). However, the work of Patrick (1986) has provided evidence that these cells lack haemagglutinating activity.

Babb and Cummins (1978) observed that on average approximately 10% of cells of Bacteroides strains produced a fibrous capsule. In our hands, 24 h cultures of bacteroides contained ca 10^9 cells/ml (Chapter 4). Therefore, if on average 10% of the cells possess a fibrous capsule, then these capsule-producing cells would be present in 10^8 cells/ml, a concentration exceeding that required for the inhibition of phagocytosis. A possible function of the capsule of Bacteroides species may, therefore, be to facilitate the colonisation process by protection from the immune system of cells capable of haemagglutination.

GENERAL DISCUSSION

GENERAL DISCUSSION

As the study progressed the complexity of each section of the work was daunting. The characterization of the test strains proved a major assignment. Storage at -70°C was by far superior to storage on agar plates as it removed the need for frequent sub-culture to maintain viability. The method of White and Sands (1985) was an efficient one which facilitated frequent use of the stored cultures. It was possible to sub-culture without thawing thereby reducing the time involved and destruction of bacterial cells caused by frequent freezing and thawing.

The mini-test system was found to be an effective way of characterizing Bacteriodes. However tests had to be repeated several times on atypical members of the three groups to confirm the results. Reading of the results was very time consuming as estimates of turbitity were required for all fermention tests. Fast-growing strains often produced equivocal results for fermentation tests and use of a pH meter was therefore required to confirm the results. It was convenient to investigate 10 - 15 strains at the same time with a mixture of fast and slow-growing strains to spread out the reading of the results.

The development of an effective method of plasmid analysis was by far the most challenging section of the study. It was developed over several months with every effort being made to improve and perfect the selected technique. The final method was suitable for the isolation and detection of plasmids covering an extensive size

range as plasmids less than 10 MDa and greater than 100 MDa were isolated. This study served to emphasize the need for the use of methods developed for the specific organisms under investigation. Established methods which gave good results with aerobes and facultative anaerobes failed to give satisfactory results with Bacteroides.

Werner et al. (1981) determined that the B. fragilis group were resistant to the effects of lysozyme but it has been used successfully by other workers (Guerry et al., 1973; Tinnell and Macrina, 1976) and this study has shown that it can be effectively used in the lysis of Bacteroides (a finding welcomed since it removed the need for unpleasant phenol extractions) The increased incidence of plasmid presence observed for the B. fragilis group may reflect strain variations. Alternatively it may be reminiscent of the increased frequency of isolation of anaerobes from sites of infection which resulted from the use of improved methods of isolation.

Antimicrobial susceptibility tests required particular care in performance and interpretation. The main problem encountered was the sheer amount of work involved, with agar dilution tests supplemented with broth dilution tests to ensure accuracy. Use of the agar dilution method, as opposed to the disk diffusion method, increased the number of strains which could be investigated at any one time. The multipoint inoculator was a very effective labour-saving device and a maximum of 18 strains could be tested per plate along with the two reference strains. It was found to be convenient to test strains in batches of 36 on four antibiotics. Preparation of dilutions for

use was the most tedious procedure but once prepared the work flowed smoothly.

The interpretation of results was in general straightforward. With the exception of erythromycin there was a definite cut-off point at the MICs (i.e. no visible growth). However, with erythromycin there was a tailing off effect and the end-point was set as the concentration at which there was significant reduction in growth.

Comparative Efficacy of Antimicrobial Agents

Chloramphenicol, clindamycin and metronidazole were all found to be very active against the Bacteroides. Of the non-beta-lactam antibiotics chloramphenicol had the highest activity as no resistant strains were observed. Resistance to clindamycin was found to be confined to the B. fragilis group. The percentage of resistant strains was sufficiently low to allow continued use of this antimicrobial agent in the treatment of bacteroides infections. These results are in agreement with those of other workers (Sutter and Finegold, 1976; Appelbaum and Chatterton, 1978; Brown and Waati, 1980; Phillips et al., 1981).

Reports of metronidazole resistance within the UK are significantly less than those from other countries. However, resistance to metronidazole, though rare, may occur with some strains (Ingham et al., 1978; Rotimi et al., 1979; Eme et al., 1983). This study has shown that resistance is not confined to the B. fragilis group. Despite the observation of Ingham et al. (1978) that metronidazole resistance may develop after long-term therapy, Willis

et al. (1978) noted that if the clinical use of metronidazole continued to be controlled then metronidazole resistance should not be a problem. The low incidence of metronidazole resistance confirm its continued effectiveness against the Bacteroides.

Imipenem was the most active of all the beta-lactam antibiotics tested. Resistant strains were not observed in any group The use of this agent within the United Kingdom as an anti-bacteroides drug is therefore recommended. The activity of the cephalosporins varied between the groups. They were found to be more active against the non-fragilis groups, due possibly in part to the substrate profile of the beta-lactamase enzymes of these groups (minimal cephalosporinase activity).

The therapeutic efficacy of the cefamycins has been associated with their resistance to hydrolysis by bacteroides beta-lactamases. Cefoxitin and moxalactam have been shown to have high activity against bacteroides but cefotetan was the least active beta-lactam antibiotic against the non-fragilis groups. Its activity against the B. fragilis groups as a whole was also poor. However the activity of cefotetan against the B. fragilis species was found to be high. This antimicrobial agent may therefore find clinical application in the treatment of some bacteroides infections.

While the B. fragilis groups possess intrinsic resistance to penicillin, resistance among the non-fragilis groups, as has been observed in this study, is emerging. This has been attributed to the production of beta-lactamases of mainly penicillinase activity.

Four mechanisms of cefoxitin resistance have been suggested for the Bacteroides. They are beta-lactamase hydrolysis (Cuchural et al., 1983), alterations in an intrinsic character [possibly cell wall change] (Dornbusch et al., 1980)] and alterations of outer membrane proteins and PBP affinities to the antimicrobial agent (Piddock and Wise, 1987). The results of this study have indicated a possible role of plasmid DNA in cefoxitin sensitivity.

Differences in the plasmid profile of resistant mutant strains compared to that of the sensitive parent strain have indicated that genes carried on plasmid DNA may make bacteroides host cells more susceptible to the effects of cefoxitin. An examination of the PBP competition assays of Piddock and Wise (1987) have shown that a possible mechanism of sensitivity is the alteration of access to the penicillin binding proteins or alteration of the affinity of proteins to cefoxitin by plasmid encoded genes.

Despite the established correlation of MLS resistance and plasmid DNA in Bacteroides (Guiney et al., 1984; Rotimi et al., 1981; Tally et al., 1979, 1982; Welch et al., 1979) conclusive results of such plasmid involvement in the strains examined were not made. However, the incidence of low-level resistance among the isolates was found to be higher than previously reported. The nature of the genetic determinants were also found to be different. While this study confirmed that low-level resistance is carried on the chromosome, the observations suggest that there may be more than one plasmid-borne high level clindamycin-resistance determinants, and

such determinants may be carried on the Bacteroides chromosome. Plasmids in Bacteroides, have not been shown to increase the sensitivity of the strains to nalidixic acid. All strains, regardless of their plasmid content, were resistant.

The Bacteroides are a suspected reservoir of antibiotic resistance plasmids (Del Bene et al., 1976; Guiney and Davis, 1975; Stiffler et al., 1974; Tinnell and Macrina, 1976; Shoemaker and Salyers, 1987). The inability to demonstrate the transfer of antibiotic resistance genes from Bacteroides to E. coli does not rule out this possibility. There are no known antibiotic resistance determinants which are expressed in both E. coli and Bacteroides, a factor which may reflect differences in plasmid replication and gene expression (Guiney et al., 1984; Shoemaker et al., 1985; 1986a; 1986b). However, determinants on transposons from Bacteroides which are expressed in E. coli are not expressed in Bacteroides and vice versa (Guiney et al., 1984; Robillard et al., 1985; Shoemaker et al., 1985; 1986). It is possible therefore that Bacteroides do in fact act as a source of resistance genes which may, due to lack of expression in the donor organisms, be difficult to identify and relate to the Bacteroides. In addition, this study has provided evidence that other mechanisms of increasing the resistance of facultative anaerobes may exist in the Bacteroides.

The observations of other workers support the theory that encapsulated strains of Bacteroides may have a protective function towards unencapsulated strains of Bacteroides and facultative anaerobes (Connolly et al., 1984; Reid and Patrick, 1984). This study has provided no evidence of an involvement between plasmid DNA

and capsule variation in Bacteroides. However, due to the small number of strains examined and the simplicity of the plasmid profiles obtained, further work is required in this area. It is possible that if capsule variation in strains with large numbers of plasmids was examined, differences in plasmid profiles may be obtained. As with other areas requiring further work, a decision had to be made to limit experimental procedures to facilitate the completion of other aspects of this study, and to prevent its indefinite extension.

The high incidence of plasmids in Bacteroides, especially among the clinically important B. fragilis group, indicates an as yet undisclosed function of this type of DNA. It is hoped that the material in this study has made a contribution towards the understanding of the role of plasmids.

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APPENDIX I

APPENDIX I The identity of bacterial strains used throughout the study.

Strain number	Bacteroides species
MH 1	B. uniformis
MH 2	B. fragilis
MH 3	"
MH 4	"
MH 5	"
MH 6	"
MH 7	"
MH 8	"
MH 9	"
MH 10	"
MH 11	"
MH 12	"
MH 13	"
MH 14	"
MH 15	
MH 16	B. ovatus
MH 17	B. fragilis
MH 18	"
MH 19	"
MH 20	"
MH 21	"
MH 22	B. thetaiotaomicron
MH 23	B. fragilis
MH 24	B. distasonis

Strain number	Bacteroides species
MH 25	B. fragilis
MH 26	B. uniformis
MH 27	B. fragilis
MH 28	"
MH 29	B. uniformis
MH 30	B. fragilis
MH 31	"
MH 32	"
MH 33	B. uniformis
MH 34	B. fragilis
MH 35	"
MH 36	"
MH 37	B. uniformis
MH 38	B. fragilis
MH 39	"
MH 40	"
MH 41	"
MH 42	"
MH 43	B. uniformis
MH 44	B. fragilis
MH 45	"
MH 46	"
MH 47	"
MH 48	"

Strain number	Bacteroides species
MH 49	B. fragilis
MH 50	"
MH 51	B. bivius
MH 52	B. fragilis
MH 53	"
MH 54	"
MH 55	B. species
MH 56	B. disiens
MH 57	B. capillosis
MH 58	B. uniformis
MH 59	B. uniformis
MH 60	B. bivius
MH 61	"
MH 62	B. species
MH 63	B. species
MH 64	B. fragilis
MH 65	"
MH 66	"
MH 67	"
MH 68	"
MH 69	"
MH 70	"
MH 71	"
MH 72	B. bivius
MH 73	B. corporis

Strain number	Bacteroides species
MH 74	B. species
MH 75	B. capillosus
MH 76	B. ovatus
MH 77	B. fragilis
MH 78	"
MH 79	B. thetaiotaomicron
MH 80	B. fragilis
MW 1	B. bivius
MW 2	"
MW 3	"
MW 4	"
MW 5	"
MW 6	"
MW 7	"
MW 8	B. disiens
MW 9	"
MW 10	B. bivius
MW 11	"
MW 12	"
MW 13	"
MW 14	"
MW 15	"
MW 16	"
MW 17	"
MW 18	B. disiens

Strain number	Bacteroides species
MW 19	B. disiens
MW 20	"
MW 21	"
MW 22	B. bivius
MW 23	B. disiens
MW 24	B. bivius
MW 25	"
MW 26	"
MW 27	"
MW 28	B. oralis
MW 29	"
MW 30	B. thetaiotaomicron
MW 31	"
MW 32	"
MW 33	"
MW 34	"
MW 35	"
MW 36	B. vulgatus
MW 37	"
MW 38	B. uniformis
MW 39	B. ovatus
MW 40	"
MW 41	B. oralis
MW 41	B. ruminicola
MW 42	B. distasonis

Strain number	Bacteroides species
MW 43	B. distasonis
MPRL 528	B. melaninogenicus
MPRL 510	B. fragilis
MPRL 628	"
MG 1	B. eggerthi
MG 1	B. asaccharolyticus
MG 6	"
MG 7	B. species
MG 8	B. asaccharolyticus
MG 10	"
MG 11	B. species
MG 13	B. uniformis
MG 14	B. vulgatus
MG 16	B. ovatus
MG 17	B. splanchnicus
MH 18	B. species
MG 19	B. ovatus
MG 20	B. putridenis
MG 23	B. asaccharolyticus
MG 24	B. species
MG 25	B. asaccharolyticus
MG 29	B. fragilis
MG 31	B. saccharolyticus
MG 33	B. bivius
MG 34	B. species

APPENDIX 2

Appendix 2 Molecular Masses of Plasmids Detected in Bacteroides Species

Strain number	Number of plasmids detected	Molecular masses of plasmids (MDa)
MH1	2	3, 2.8, 1.7, 1.3
2	1	3.3
3	7	33, 15, 3, 2.4, 1.75, 1.15
4	7	42, 19, 5.7, 3.8, 2.5, 1.4, 2.2
5	1	1.35
6	2	3, 1.7
7	4	3.2, 2.5, 1.65, 1.35
8	2	2.5, 1.3
9	2	2.2, 1.35
10	-	
11	1	3.2
12	2	3.2, 1.5
13	5	39, 3.2, 2.35, 1.6, 1.34
14	1	1.35
15	2	3.4, 1.65
16	1	59
17	5	42, 3, 2.8, 2.45, 1.45
18	1	1.4
19	5	19, 3.3, 2.3
20	-	
21	4	3.4, 2.3, 1.6, 1.35
22	4	2.8, 2.4, 2.2, 1.1
23	3	3.3, 1.6, 1.4

(continued)

Strain number	Number of plasmids detected	Molecular masses of plasmids (MDa)
MH24	-	
25	1	2.4
26	6	32, 13.6, 4.2, 3.1, 2.25, 1.3
27	-	
28	-	
29	-	
30	2	3.3, 1.65
31	3	2.3, 1.7, 1.45
32	3	24.7, 10.4, 2.4, 2.0
33	-	
34	1	2.5
35	1	108
36	2	5.4, 3.1
37	4	5.4, 3.1, 3.0, 2.2
38	6	19.4, 10.5, 2.5, 2.3, 1.7, 1.45
39	2	27, 10.1
40	-	
41	-	
42	-	
43	-	
44	10	
45	1	1.7
46	1	1.7

(continued)

Strain number	Number of plasmids detected	Molecular masses of plasmids (MDa)
MH47	-	
48	6	4.3, 3.9, 3.0, 1.95, 1.8, 1.7
49	-	
50	-	
51	-	
52	5	16, 4.6, 3.8, 1.6, 1.1
53	-	
54	4	43, 2.2, 1.6, 1.25
55	-	
56	-	
57	-	
58	-	
59	-	
60	-	
61	-	
62	-	
63	-	
64	6	5.4, 4.6, 3.4, 2.05, 1.9, 1.65
65	-	
66	-	
67	-	
68	6	3.8, 3.4, 2.25, 1.8, 1.7, 1.4
69	-	
70	-	

(continued)

Strain number	Number of plasmids detected	Molecular masses of plasmids (MDa)
MH71	-	
72	-	
73	-	
74	-	
75	-	
76	2	1.75, 1.1
77	-	
78	2	2.4, 1.65
79	-	
80	9	17.5, 13.5, 4.7, 3.9, 3.5, 2.4, 1.6, 1.3
MW1	-	
2	-	
3	-	
4	-	
5	-	
6	-	
7	-	
8	-	
9	-	
10	-	
11	-	
12	-	
13	-	

APPENDIX 3

APPENDIX 3 Susceptibility of Bacteroides species to antimicrobial agents

Metronidazole	Mz	Penicillin	PEN	Cefoxitin	CFX
Chloramphenicol	Cm	Cefuroxime	CXM	Moxalactam	MOX
Clinidamycin	Cln	Cefotaxime	CTX	Imipenem	IMP
Erythromycin	Erm	Cefotetan	CTN	Nalidixic acid	NAL

(i) Bacteroides fragilis strains.

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MH 2	4	0.5	>64	>128	2	8	32	64	4	>128	0.5	>128
MH 3	2	0.5	<0.25	16	<0.25	4	8	4	0.5	8	<0.25	128
MH 5	4	2	<0.25	2	0.25	8	16	8	4	16	0.5	>128
MH 6	4	2	0.25	4	0.25	4	8	4	2	64	<0.25	128
MH 7	4	4	0.25	4	0.25	4	16	2	0.5	16	0.5	128
MH 8	4	4	<0.25	32	<0.25	8	8	8	2	32	0.5	>128
MH 9	4	2	<0.25	8	0.25	4	16	16	8	32	<0.25	128
MH 10	2	1	1	8	1	2	8	16	8	4	<0.25	128
MH 11	4	1	16	8	0.25	4	16	4	4	16	<0.25	128

(i) Bacteroides fragilis strains (continued)

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MH 12	4	2	>64	>64	0.5	8	32	32	4	>128	0.5	128
MH 13	4	4	<0.25	4	<0.25	4	8	8	4	32	0.25	128
MH 14	4	1	0.25	4	0.5	4	16	8	4	16	0.5	128
MH 15	4	0.5	0.25	16	0.25	8	8	16	4	32	0.5	>128
MH 17	4	0.5	<0.25	32	<0.25	4	8	4	1	16	0.25	128
MH 18	4	2	0.25	32	0.25	4	8	16	4	16	0.5	>128
MH 19	2	2	<0.25	8	0.25	8	16	8	2	16	0.25	128
MH 20	4	1	0.25	32	<0.25	4	8	4	1	16	0.25	128
MH 21	4	0.5	0.25	8	<0.25	4	8	4	0.5	16	0.26	128
MH 23	4	4	0.25	32	<0.25	4	8	8	1	32	0.5	>128
MH 25	2	1	0.5	8	0.5	4	16	8	1	2	<0.25	128
MH 27	4	0.5	0.5	4	1	4	16	64	32	128	0.5	128
MH 28	2	1	<0.25	4	<0.25	4	8	4	2	16	0.5	128

(i) Bacteroides fragilis strains (continued)

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MH 30	4	1	0.5	8	0.5	4	16	8	4	32	<0.25	>128
MH 31	4	2	0.25	4	0.5	1	32	8	2	32	<0.25	128
MH 32	4	0.5	<0.25	32	0.25	4	16	32	4	32	0.25	128
MH 34	4	1	<0.25	32	0.25	4	8	32	4	32	0.25	>128
MH 35	4	4	0.25	8	32	16	>128	8	1	32	4	128
MH 36	4	2	<0.25	4	<0.25	4	8	16	4	32	0.5	128
MH 38	4	0.5	1	16	<0.25	4	>128	8	4	16	0.5	>128
MH 39	4	2	0.25	8	0.5	4	8	8	4	32	0.25	>128
MH 40	4	4	<0.25	32	<0.25	4	8	16	2	16	0.5	128
MH 41	4	0.5	0.25	32	0.25	8	16	8	2	32	0.5	128
MH 42	4	2	<0.25	4	0.5	4	8	8	16	32	1	128
MH 44	4	2	<0.25	4	0.25	1	16	4	1	16	<0.25	128
MH 45	4	0.5	0.25	32	0.25	4	16	8	1	>128	0.5	128

(i) Bacteroides fragilis strains (continued)

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MH 46	4	0.5	0.25	32	0.25	4	8	8	2	32	1	128
MH 47	4	2	0.25	8	4	4	16	64	128	>128	0.5	128
MH 48	4	2	<0.25	64	4	4	8	64	128	>128	0.5	>128
MH 49	4	2	0.25	8	2	4	8	64	128	>128	0.5	>128
MH 50	4	0.5	<0.25	8	0.25	4	8	8	2	32	0.25	128
MH 52	4	1	0.25	32	4	4	8	128	128	>128	0.5	>128
MH 53	4	0.5	0.25	32	0.25	4	8	8	2	16	0.25	>128
MH 54	4	2	<0.25	2	0.25	4	8	8	4	32	0.25	>128
MH 64	2	1	<0.25	2	0.25	8	16	4	1	8	0.5	128
MH 65	4	2	0.25	8	4	4	8	64	128	>128	1	>128
MH 66	4	<0.25	<0.25	32	0.25	4	8	32	4	32	0.5	>128
MH 67	2	<0.25	0.5	16	16	128	8	16	128	>128	16	>128
MH 68	4	2	128	128	2	16	64	16	16	32	0.5	>128

(i) Bacteroides fragilis strains (continued)

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MH 46	4	0.5	0.25	32	0.25	4	8	8	2	32	1	128
MH 47	4	2	0.25	8	4	4	16	64	128	>128	0.5	128
MH 48	4	2	<0.25	64	4	4	8	64	128	>128	0.5	>128
MH 49	4	2	0.25	8	2	4	8	64	128	>128	0.5	>128
MH 50	4	0.5	<0.25	8	0.25	4	8	8	2	32	0.25	128
MH 52	4	1	0.25	32	4	4	8	128	128	>128	0.5	>128
MH 53	4	0.5	0.25	32	0.25	4	8	8	2	16	0.25	>128
MH 54	4	2	<0.25	2	0.25	4	8	8	4	32	0.25	>128
MH 64	2	1	<0.25	2	0.25	8	16	4	1	8	0.5	128
MH 65	4	2	0.25	8	4	4	8	64	128	>128	1	>128
MH 66	4	<0.25	<0.25	32	0.25	4	8	32	4	32	0.5	>128
MH 67	2	<0.25	0.5	16	16	128	8	16	128	>128	16	>128
MH 68	4	2	128	128	2	16	64	16	16	32	0.5	>128

(i) Bacteroides fragilis strains (continued)

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MH 70	4	2	0.25	8	0.25	4	8	8	2	8	1	128
MH 71	4	2	<0.25	8	0.25	8	8	8	2	32	0.25	128
MH 77	4	1	0.25	4	<0.25	4	16	4	2	16	<0.25	128
MH 78	4	<0.25	0.25	8	<0.25	16	32	32	2	32	1	128
MH 80	4	<0.25	0.25	16	0.25	8	16	16	2	64	0.25	128
MG 29	2	1	<0.25	2	<0.25	8	>128	8	4	4	2	128
MRPL 610	4	32	<0.25	4	0.5	16	64	8	2	32	0.5	>128
MPRL 628	4	16	0.25	2	0.5	8	32	8	1	16	0.5	>128

(ii) Bacteroides fragilis Group (excluding Bacteroides fragilis)

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MH 1	4	4	0.25	8	16	16	>128	16	8	16	0.25	128
MH 4	2	0.25	<0.25	4	0.5	4	16	2	2	8	<0.25	128
MH 16	4	4	2	8	8	16	128	32	8	32	0.5	128
MH 22	4	1	0.5	16	16	16	128	128	32	32	0.5	128
MH 24	4	1	0.25	2	32	16	>128	4	1	64	1	>128
MH 26	4	2	0.5	4	4	8	128	16	16	32	0.5	128
MH 29	8	4	128	128	16	16	128	16	16	64	0.5	128
MH 33	2	0.25	0.25	4	1	2	31	>128	16	4	<0.25	128
MH 37	4	2	0.5	8	2	8	64	32	8	32	0.5	128
MH 43	4	2	<0.25	8	0.5	2	32	128	4	64	0.5	>128
MH 58	4	4	16	128	2	16	64	16	16	32	0.5	>128
MH 59	4	2	16	128	2	8	128	16	16	32	0.25	128
MH 76	4	4	0.25	4	2	8	128	32	8	16	1	128

(ii) Bacteroides fragilis Group (excluding Bacteroides fragilis)

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MH 79	4	4	128	128	2	8	64	32	16	16	1	128
MG 1	1	1	0.5	2	<0.25	2	64	32	16	4	1	128
MG 13	4	2	0.5	1	2	8	128	64	64	4	0.5	128
MG 14	1	1	<0.25	2	0.25	2	64	0.2	2	2	0.5	128
MG 16	4	4	0.5	16	4	8	32	32	4	4	0.5	128
MG 17	2	1	<0.25	2	0.25	1	2	32	2	0.25	0.5	32
MG 19	4	2	<0.25	16	4	8	32	32	4	16	0.5	128
MW 30	4	2	0.25	16	16	16	64	16	16	16	0.25	128
MW 31	4	2	0.25	8	8	16	64	32	16	16	0.25	128
MW 32	8	4	0.5	16	16	2	64	16	16	16	<0.25	128
MW 33	8	2	2	8	2	8	32	2	16	16	<0.25	>128
MW 34	2	4	0.25	4	64	16	>128	32	8	16	0.25	>128

(ii) Bacteroides fragilis Group (excluding Bacteroides fragilis)

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MW 35	4	2	0.25	16	4	32	128	2	8	16	<0.25	>128
MW 36	2	<0.25	<0.25	4	0.5	4	16	4	2	4	<0.25	128
MW 37	4	1	<0.25	2	2	2	64	1	8	1625	<0.25	128
MW 39	4	2	0.25	8	4	8	64	32	4	16	0.5	128
MW 40	4	2	0.25	8	8	8	32	32	8	16	0.5	128
MW 43	8	1	0.25	4	16	16	64	4	16	32	0.5	128

(iii) melaninogenicus/oralis strains.

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MH 51	1	2	0.25	0.5	0.5	0.5	4	1	0.25	1	0.25	64
MH 56	0.5	2	<0.25	1	<0.25	0.5	4	<0.25	1	1	<0.25	64
MH 60	1	4	<0.25	0.25	4	2	64	32	2	16	0.25	128
MH 61	1	4	<0.25	0.25	4	4	32	32	2	4	<0.25	64
MH 72	2	2	<0.25	1	0.25	0.5	4	0.5	0.25	2	0.25	64
MH 73	1	0.5	<0.25	0.25	4	1	16	4	0.5	4	<0.25	128
MW 1	2	2	<0.25	2	0.5	0.5	2	0.5	<0.25	<0.25	<0.25	128
MW 2	1	1	<0.25	0.5	<0.25	0.5	8	4	0.5	0.5	0.5	128
MW 3	1	4	<0.25	0.5	1	<0.25	8	1	0.5	0.5	<0.25	128
MW 4	1	1	<0.25	0.5	<0.25	<0.25	1	1	<0.25	0.25	0.25	32
MW 5	0.5	1	<0.25	0.5	1	1	8	4	<0.25	0.5	<0.25	64
MW 6	1	4	<0.25	0.5	16	4	64	16	2.0	32	<0.25	128
MW 7	1	1	<0.25	0.5	4	0.5	16	4	0.5	1	0.25	128

(iii) melaninogenicus/oralis strains (continued).

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MW 8	1	1	<0.25	2	0.5	0.25	4	4	<0.25	1	<0.25	128
MW 9	2	1	<0.25	0.25	2	0.5	4	0.5	<0.25	4	<0.25	64
MW 10	0.5	4	<0.25	0.5	2	0.25	8	16	1	2	2	64
MW 11	1	4	<0.25	0.5	1	0.5	4	32	8	2	<0.25	128
MW 12	0.5	2	<0.25	0.5	1	0.25	4	2	<0.25	<0.25	<0.25	128
MW 13	0.5	2	<0.25	0.5	1	0.25	8	4	0.25	1	<0.25	128
MW 14	0.5	1	<0.25	0.5	1	0.5	4	2	0.5	2	<0.25	64
MW 15	1	2	0.5	0.25	4	4	32	2	0.5	8	<0.25	128
MW 16	1	1	<0.25	0.5	4	1	16	4	0.25	2	<0.25	64
MW 17	1	0.5	<0.25	1	1	0.25	8	2	4	<0.25	<0.25	64
MW 18	1	0.5	<0.25	1	0.25	2	8	2	2	2	<0.25	64
MW 19	0.5	2	<0.25	1	0.5	2	8	2	<0.25	2	2	32
MW 20	0.5	1	<0.25	0.5	<0.25	0.25	8	2	0.25	2	0.5	128

(iii) melaninogenicus/oralis strains (continued).

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MW 21	1	0.5	<0.25	<0.25	<0.25	0.5	16	0.25	<0.25	2	<0.25	32
MW 22	1	1	<0.25	0.25	2	2	16	4	2	4	<0.25	64
MW 23	0.5	1	<0.25	0.5	4	2	32	32	4	4	<0.25	64
MW 24	0.5	4	<0.25	1	4	0.25	16	16	4	2	<0.25	64
MW 25	4	0.5	1	1	8	16	64	4	4	16	<0.25	>128
MW 26	0.5	2	<0.25	0.5	1	<0.25	8	2	<0.25	0.5	<0.25	64
MW 27	1	0.25	<0.25	0.25	<0.25	0.25	2	0.25	<0.25	<0.25	<0.25	64
MW 28	1	1	<0.25	2	1	0.25	8	0.25	1	1	<0.25	64
MW 29	1	2	<0.25	2	1	<0.25	8	0.25	0.5	0.5	<0.25	64
MW 41	0.5	0.5	<0.25	<0.25	0.25	0.25	1	0.25	<0.25	<0.25	<0.25	128
MW 42	1	1	<0.25	1	1	<0.25	16	1	<0.25	1	<0.25	64
MG 33	1	2	<0.25	1	1	1	8	2	<0.25	2	<0.25	128
MG 37	1	2	<0.25	1	1	<0.25	1	4	<0.25	<0.25	<0.25	32

(iii) melaninogenicus/oralis strains (continued).

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MG 41	2	0.5	<0.25	16	4	8	32	32	8	16	<0.25	128
MPRL 528	1	16	<0.25	0.25	8	2	16	4	4	8	<0.25	64

(iv) Asaccharolytic Bacteroides strains.

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MG 2	0.5	0.25	<0.25	<0.25	<0.25	0.25	1	8	<0.25	<0.25	<0.25	128
MG 6	1	1	<0.25	<0.25	0.25	0.25	0.5	0.25	0.25	<0.25	<0.25	32
MG 7	0.5	1	<0.25	1	0.5	2	8	2	<0.25	2	2	32
MG 8	2	<0.25	<0.25	<0.25	0.25	0.25	0.25	1	0.25	<0.25	<0.25	128
MG 10	1	1	<0.25	<0.25	<0.25	0.25	0.25	0.25	<0.25	<0.25	<0.25	64
MG 11	<0.25	0.5	<0.25	<0.25	0.5	<0.25	0.25	0.25	0.5	<0.25	0.5	128
MG 18	0.5	1	<0.25	<0.25	<0.25	0.5	0.5	0.25	0.25	<0.25	0.5	64
MG 20	2	2	0.25	8	0.125	8	16	4	4	8	0.5	128
MG 23	0.5	1	<0.25	<0.25	0.5	0.25	1	0.25	<0.25	<0.25	<0.25	32
MG 24	1	2	<0.25	1	0.5	<0.25	1	0.5	<0.25	<0.25	<0.25	32
MG 25	0.5	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	32
MG 31	0.5	<0.25	<0.25	<0.25	<0.25	<0.25	0.25	0.25	<0.25	<0.25	32	32

(iv) Asaccharolytic Bacteroides strains (continued).

Strain Number	Minimum Inhibitory Concentration (mg/l)											
	Cm	Mz	Cln	Erm	MOX	CFX	CTN	CXM	CTX	PEN	IMP	NAL
MG 34	4	<0.25	<0.25	0.5	8	4	64	16	16	<0.25	<0.25	64
MG 35	1	0.25	<0.25	0.5	<0.25	<0.25	0.25	0.5	<0.25	<0.25	<0.25	64
MG 38	0.5	<0.25	<0.25	<0.25	<0.25	<0.25	0.25	0.25	<0.25	<0.25	32	32
MG 40	1	<0.25	<0.25	<0.25	<0.25	<0.25	0.25	0.25	<0.25	<0.25	<0.25	32
MH 55	1	0.25	<0.25	<0.25	<0.25	0.25	1	0.25	1	0.25	<0.25	64
MH 57	1	0.5	<0.25	0.5	0.5	0.25	1	2	0.25	<0.25	0.25	64
MH 62	1	<0.25	<0.25	0.5	<0.25	1	0.5	1	0.5	<0.25	0.25	64
MH 63	0.5	0.25	<0.25	0.25	0.25	0.25	0.25	0.25	2	<0.25	<0.25	64
MH 74	0.5	0.5	<0.25	<0.25	0.25	0.5	0.5	0.25	<0.25	<0.25	<0.25	64
MH 75	0.25	<0.25	<0.25	0.5	<0.25	0.25	0.25	0.25	0.25	<0.25	0.5	64

APPENDIX 4

APPENDIX 4 Reagents used for polyacrylamide gel electrophoresis (PAGE)

Electrode Buffer (Final strength)

0.025 M Tris

0.192 M glycine

0.1% sodium dodecyl sulphate (SDS)

Separating Gel Buffer (Double strength)

0.75 M Tris-HCl pH 8.8

0.2% SDS

Stacking Gel Buffer (Double Strength)

0.25 M Tris-HCl pH 6.8

0.2% SDS

Sample Buffer (single strength)

0.0625 M Tris-HCl pH 6.8

2% SDS

10% glycerol

1% 2-mercaptoethanol

0.001% bromophenol blue

Acrylamide Stock Solution

40% acrylamide

1.08% methylenebisacrylamide

PAGE Cell Washing Buffer

0.05 M sodium phosphate buffer pH 7.4

0.15 M NaCl

PAGE EDTA Buffer

0.05 M sodium phosphate buffer pH 7.4

0.15 M NaCl

0.01 M EDTA di-sodium salt

SDS-PAGE

Coomassie blue stain

Solution 1

500 mg	Coomassie blue (Bio-rad R-250 or Gurr Brilliant blue R)
250 ml	Propan-2-ol (BDH GPR)
100 ml	Glacial acetic acid (BDH Analar)
650 ml	Distilled water

Solution 2

50 mg	Coomassie blue (Bio-Rad R-250 or Gurr Brilliant blue R)
100 ml	Propan-2-ol (BDH GPR)
100 ml	Glacial acetic acid (BDH Analar)
800 ml	Distilled water

Solution 3

24 mg	Coomassie blue (Bio-Rad R-250 or Gurr Brilliant blue R)
100 ml	Glacial acetic acid (BDH Analar)
900 ml	Distilled water

Solution 4

100 ml	Glacial acetic acid (BDH Analar)
400 ml	Methanol (BDH GPR)
500 ml	Distilled water

Solution 5

200 ml Glacial acetic acid (BDH Analar)

1800 ml Distilled water