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THE BIOLOGICAL AND STRUCTURAL CHARACTERIZATION OF TRANSFERABLE BACTEROIDES R PLASMIDS

Rodney A. Welch

THE BIOLOGICAL AND STRUCTURAL CHARACTERIZATION OF TRANSFERABLE *BACTEROIDES* R PLASMIDS

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Rodney A. Welch
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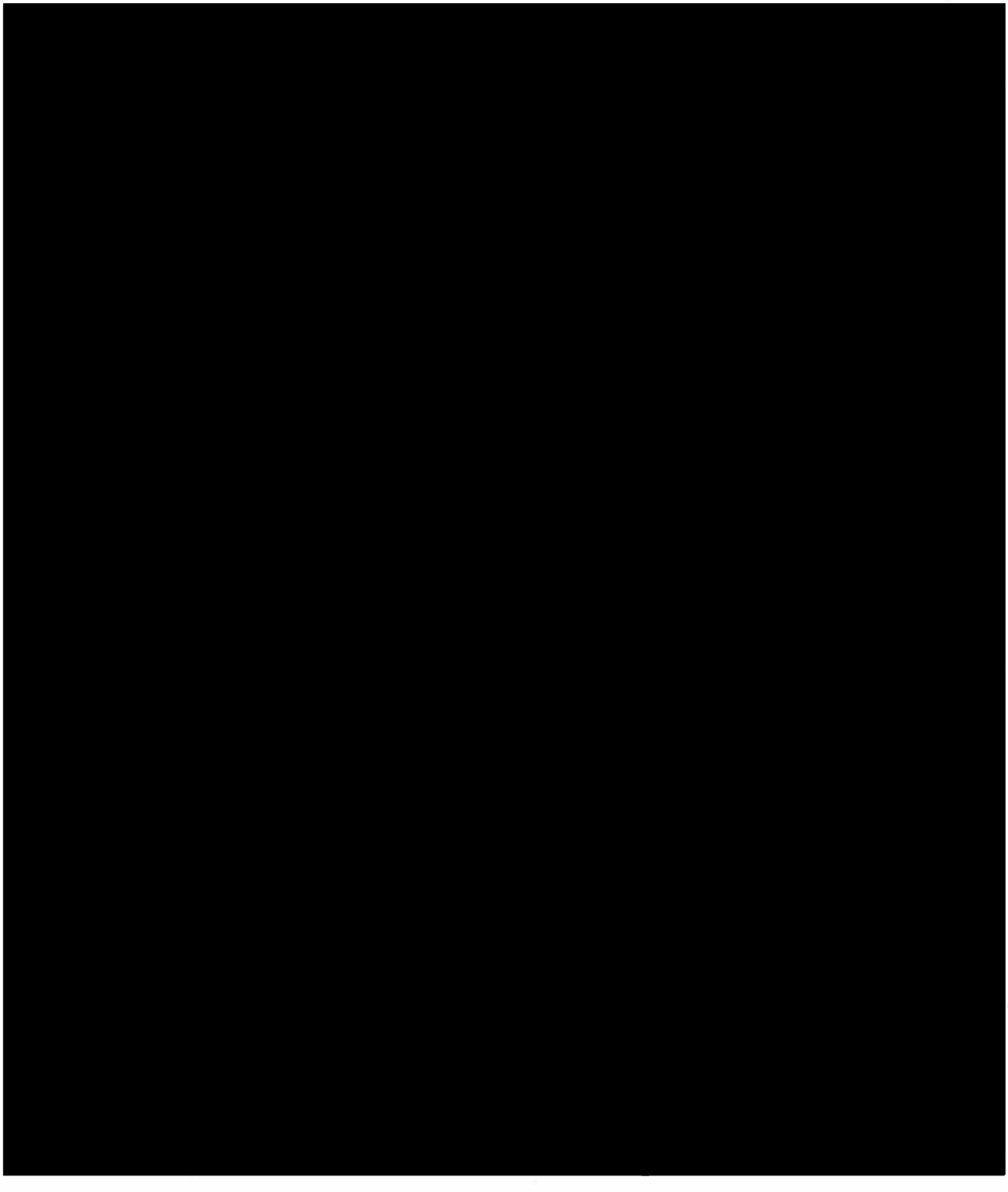

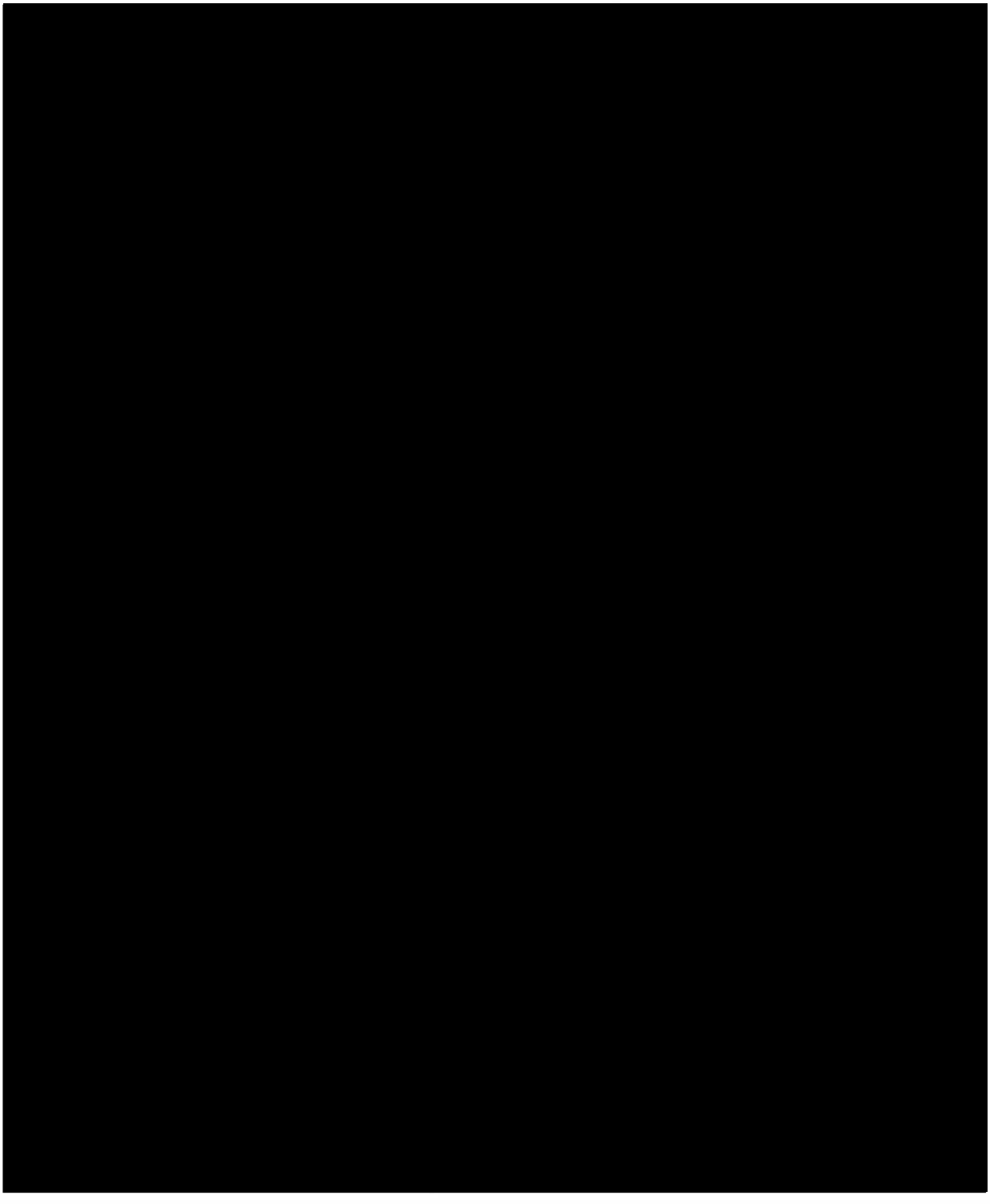

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF TABLES

TABLE OF TABLES (Continued)

TABLE OF FIGURES

ABBREVIATIONS

Ap	ampicillin
Cc	clindamycin
CCC	covalently closed circular
CFU	colony forming units
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOC	deoxycholate
EB	ethidium bromide
EDTA	ethylenediamine tetraacetate
Em	erythromycin
E.O.P.	efficiency of plating
G+C	guanosine plus cytosine
LM	lincosamide-macrolide
Ln	lincomycin
Mdal	10^6 daltons
M.I.C.	minimum inhibitory concentration
PHC	penassay heme cysteine
Rf	rifampicin
RNase	ribonuclease
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
Tc	tetracycline
t-RNA	transfer ribonucleic acid
VPI	Virginia Polytechnic Institute

REVIEW OF LITERATURE

Introduction

In this literature review I will briefly describe the general biology of transferable antibiotic resistance in bacteria, the genetic elements involved (plasmids) and several specific plasmid-associated phenotypes. This will be followed with a review of the general biology of *Bacteroides*, the information known concerning its plasmid reservoir and finally a review of the information documenting the emergence of transferable clindamycin resistance in *Bacteroides*. The latter aspect represents the central focus of research described in this thesis.

Transferable Antibiotic Resistance: General Considerations

In recent years it has come to light that the use of antibiotics in the treatment of bacterial diseases is a double edged sword. Without doubt, we are far better off as a society with the use of antibiotics. However, we have had to come to the realization that bacteria like other living creatures will obey Darwin's law of natural selection in response to antibiotics.

The discovery of transferable multiple antibiotic resistance is generally credited to the Japanese workers,

Ochiai and Akiba (25,102). Independently in 1959 they carried out experiments in which mutiple antibiotic resistant Escherichia coli cells were mixed with antibiotic sensitive Shigella cells. Following their incubation together it was possible to identify Shigella cells which now possessed the same multiple antibiotic resistance pattern as the E. coli. Subsequent observations by other Japanese workers delineated that conjugation was the genetic event involved in the resistance transfer (25,102). Mitsuhashi is credited with naming the property of transmissable antibiotic resistance as the R factor. This term was obviously based on the earlier precedent established with the property of transferable phenotypic traits being attributable to the E. coli K12 F factor [F for fertility](37).

Both the transfer of F factor and R factors occur by the bacterial genetic process termed conjugation. This event was initially discovered by Lederberg and Tatum (57). Conjugation is strictly dependent on direct cell to cell contact between donor and recipient cells (21). Experimentally this genetic event can be differentiated from bacterial transformation (uptake and expression of naked DNA) and transduction (transfer mediated by bacteriophage) by showing that donor cell-free filtrates or donor cell extracts cannot effect transfer of whatever phenotypic trait is being acquired by recipient cells. Additionally the dependence on direct cell to cell contact is usually demon-

strated by devising an experimental system where donor and recipient cells share the same liquid culture medium but where they are physically separated by filters (21).

The molecular nature of the F and R factors eluded workers for several years following the characterization of their biological properties. It was clear that the factors were transferable at frequencies far greater than any associated transfer of chromosomal determined phenotypes (42,103). Secondly, they could be picked up by transducing viruses as independent linkage groups apart from any linkage with chromosomal phenotypes (2,105). From these results, it seemed certain that the F and R factors existed within the bacterial cell as extrachromosal elements or supernumerary chromosomes. It was also clear that these genetic elements were dispensible since F- cells and cells "cured" of their R factors were physiologically stable. (42,104). The direct demonstration that the F factor was in fact DNA was accomplished by Marmur et al (66). In those experiments they were able to detect the acquisition of a new DNA species subsequent to F factor transfer from E. coli to Serratia marcescens. By equilibrium density ultracentrifugation, they detected in the F+ Serratia an additional DNA species with a density different than that of the host DNA. The density of the new DNA species was similar to the density of the donor E. coli DNA. This same approach permitted Falkow et al to detect the transfer

of R factor DNA from E. coli to Proteus mirabilis (26). Freifelder and Freifelder were the first to suggest that extrachromosomal genetic elements were circular DNA molecules (32). The unequivocal evidence of their circular nature was seen in electron micrographs of purified R factor DNA acquired by Clowes' group (76). Additionally Clowes also demonstrated that one of the physical forms for the circular DNA was the supercoiled or supertwisted state (76).

Bacterial Plasmids: Molecular Nature

The term plasmid was coined by Joshua Lederberg long before anyone had a clue as to their physical state. He defined it as any extranuclear structure capable of autonomous reproduction within the bacterial cell (56). Now we know bacterial plasmids exist as extrachromosomal covalently closed circular DNA duplexes which replicate in a physically autonomous fashion from the chromosomal DNA molecule. When they are isolated from bacterial cells they are found to be predominantly in a supertwisted tertiary structure which is a feature common to all known circular duplex DNA molecules. Upon storage or limited DNase treatment it is possible to observe two additional physical forms. A single break in any of the phosphodiester bonds will result in a nicked or open circular form. This occurs because the supertwisted DNA duplex now can release torsional stress

about a free axis of rotation.

Plasmids can vary in molecular size from approximately $1x10^6$ daltons (1 Mdal) to greater than 250 Mdal (25). One criterion used in classifying plasmids is the number of copies of the plasmid per chromosomal equivalent present within the cell. Most of the known larger plasmids (>25 Mdal) are present only in a few copies per cell. The smaller plasmids are often present in numbers as high as 40 copies per cell. Plasmids can represent several percent of the entire DNA content of a cell (25). More than a single plasmid species can be found in a bacterial cell. Strains of E. coli have been isolated which stably maintain as many as eight different plasmid species of differing sizes (62).

Plasmids are autonomous units of DNA replication. Therefore they fit Jacob's definition of a replicon and most probably fit the associated replicon model. Namely there are two entities required for autonomous units of replication. They are a replicase [or initiator] and an origin of replication [the operator of replicator](43). The greatest amount of work performed on plasmid replication has been done with the E. coli plasmids Col EI and R6K. In the case of Col EI it is known that replication begins at a unique origin and proceeds in an undirectional manner (61). R6K on the other hand has two different origins where replication may begin at either or both sites simultaneous-

ly. (16). The other steps in plasmid replication are essentially the same as those seen in the synthesis of any DNA molecule. There is a need for an RNA primer and the elongation process appears to be a discontinuous process (90).

The dependency of plasmid replication on chromosomal gene products and its sensitivity to different inhibitors are two areas where different plasmids have different properties. For instance, most small E. coli plasmids (e.g., Col EI) will continue to replicate once protein synthesis is prevented by chloramphenicol treatment. However, larger plasmids (e.g., R222) will cease replication very soon after the addition of chloramphenicol (25).

Bacterial Plasmids: Associated Phenotypes

In well studied plasmid systems where extensive genetic mapping has been performed, the total proportion of genetic information devoted to the replicative functions is relatively small. A large proportion of plasmid DNA has been found to encode for what could be termed adaptive functions. It is really these adaptive functions which brought plasmids to the attention of microbiologists and biochemists. Table 1 is a representative summary of significant plasmid encoded phenotypes. It should be noted that combinations of these and similar phenotypes are also possible on a single plasmid species. At this point in the literature review it would be appropriate to focus specifically on

Table 1

Plasmid Phenotypes

Phenotype	Representative	Remarks	Reference
fertility	F, R6K	Conjugal donor ability	(25)
surface exclusion	F	Reduced ability to conjugationally transfer a plasmid to a recipient cell already harboring a closely related plasmid	(25)
incompatability	F	The unstable maintenance and segregation of two closely related plasmid species within a cell	(25)
antibiotic resistance	pSC101	Resistance to tetracycline	(14)
bacteriocin	CloDF13	Synthesis of protein with antibacterial action but generally specific for a narrow range of closely related organisms	(100)
antibiotic production	SCP1	Synthesis of broad range antibiotic materials	(111)
toxin production	Ent	Synthesis of enterotoxins	(91)
plant tumorgenecity	TI	Enables plant pathogen Agrobacterium tumefaciens to cause crown gall tumors	(17)
catabolic activity	Oct, Cam	Enzymatic ability to acquire energy from degradation of such hydrocarbons as octane and camphor	(11)

two classes of plasmid phenotypes especially germane to the research involved in this thesis: bacterial fertility and plasmid-mediated antibiotic resistance. Lincosamide-macrolide and tetracycline resistance will be described in some detail.

Bacterial Fertility

Conjugative ability enables a plasmid to mediate its own physical transfer and in some instances transfer of other plasmids or portions of the donor chromosome to a recipient cell. As mentioned before, the conjugational process is dependent on cell to cell contact but unlike classic zygote formation only a single strand of donor DNA enters with no cytoplasmic mixing occurring (19). At least in the Enterobacteriaceae, surface appendages (donor pili) encoded by the conjugative plasmid aid specific pair formation and may take part in the actual tranfer process. At present the best studied conjugative plasmid is the F factor or *E. coli*. For this element, seventeen plasmid-linked genes have been identified which are necessary for its transfer. Most of the genes appear to encode for proteins involved in pili synthesis; it is felt that many of the genes involved in the later stages of the transfer have yet to be identified (25).

Plasmids displaying conjugative ability have been found in numerous genera ranging from members of the

Enterobacteriaceae, Pseudomonads, Neisseriae, Agrobacter, Vibriio and Bacteroides to gram positive genera such as Streptomyces, Clostridia and Streptococcus (8,24,25,110). As a rule, most conjugative plasmids have a limited range of infectivity--something amounting to an intrageneric range. However, there are examples of plasmids with wide intergeneric host ranges. The archetype wide host range plasmid is RP1 which was originally found in Pseudomonas aeruginosa and known to be transmissable to Enterobacteriaceae, Pseudomonadaceae,Rhizobium, Azotobacter and Acinetobacter (9,80).

With the exception of the classic F factor(F), most plasmids having conjugative ability are repressed for the conjugative function and their transfer occurs at a frequency of only once per 100,000 (10^{-5}) to 1,000,000 (10^{-6}) donor cells (26). Ideally, the transfer of the F factor approaches 100%. In a manner poorly understood, F will promote the transfer of a portion of the chromosome to a recipient at a frequency of 10^{-5} to 10^{-6} recombinants per donor cell. In rare instances it is possible to isolate clones of F containing cells where the frequency of chromosome transfer is very high (~1%). On a molecular basis these high frequency of recombination (Hfr) clones represent the instance where the F factor has integrated into the chromosome and subsequently permits an enhanced means of chromosome transfer. In the case of transmissable R plasmids and other conjugative plasmids, the chromosome transfer is rare but detectable

at a frequency of 10^{-8} to 10^{-9} recombinants per donor cell. It is possible to isolate mutants of transmissable R factors which promote their own transfer and subsequent chromosomal mobilization at enhanced frequencies amounting to 100 to 1,000% better transfer (25).

Mechanisms of Plasmid Mediated Antibiotic Resistance and Transposition

Plasmid encoded resistance genes have been found to occur for nearly every family of antibiotics. It is not within the scope of this review to describe the mechanisms for resistance to all the different classes of antibiotics. Table 2 is a summation of some of the known enzymatic mechanisms for antibiotic resistance that are plasmid-mediated.

A great deal of the present plasmid research is focused on transposable DNA elements. They represent cases of unusual recombinational events where specific antibiotic resistance genes move as physically well-defined and discrete segments of DNA from one site on a replicon to other sites on the replicon or to sites on a different replicon present within the cell (9,13,50,53). The discrete genetic entities have been given the name transposons and their movement transposition. Tranposons facilitate the spread of antibiotic resistance from one cell to another by moving into replicons such as conjugative plasmids and bacteriophage genomes which

Table 2

Mechanisms of Plasmid-Mediated Antibiotic Resistance in Bacteria (4)

Antibiotic family	Mechanism
Penicillin	Enzymatic hydrolysis of the β-lactam ring present on penicillin
Cephalosporin	Same as above
Chloramphenicol	Inactivation of the antibiotic by direct structural alteration: acetylation of the chloramphenicol molecule
Aminoglycoside	Inactivation of the antibiotic: acetylation, adenylation or phosphorylation of the aminoglycoside molecule
Tetrycycline	Not understood but probably involves alteration in cell membrane resulting in increased impermeability of tetracycline or possibly enhanced export of tetracycline from the cell.
Macrolide	Alteration of the drug's target site; methylation of ribosomal RNA resulting in an inhibition of macrolide binding to the ribosome. This mechanism results in cross resistance to lincosamide and streptogramin B antibiotics.

serve as vehicles of genetic exchange. Resistance genes for ampicillin, sulfonamides, kanamycin, tetracycline and chloramphenicol have been found to be present as transposable genetic elements (9). There are a number of very good reviews detailing the biological and physical nature of antibiotic resistance transposons and their clinical impact (9,13,50,53).

Since plasmid-mediated resistance to clindamycin, erythromycin and tetracycline is the focus of my thesis research, the literature concerning these resistances will be described in more detail.

Plasmid-Mediated Clindamycin-Erythromycin Resistance

Clindamycin and erythromycin are two protein synthesis inhibitors which belong to different structural classes of antibiotics, respectively the lincosamide and macrolide families. However, plasmid encoded resistance to these antibiotics represents the unusual case where a single enzymatic event results in cross resistance to the two different classes of antibiotics (107). It should be noted that a third antibiotic class is also involved in this resistance event namely streptogramin B, but the antibiotic has virtually no clinical use. The precise molecular event resulting in the macrolide-lincosamide resistance is the N^6-dimethylation of adenine of the 23S rRNA (33,108). Subsequent to this event, the affinity between the antibiotics

and the ribosome is reduced. This implies that the active or binding sites for both antibiotics are probably in close juxtaposition.

The first suggestion that the resistance to both erythromycin and lincomycin was a plasmid borne trait was published by Mitsuhashi and co-workers (72). He found that the treatment of cultures with a plasmid curing agent (acriflavin) resulted in loss of both erythromycin and lincosamide resistances simultaneously. This genetic linkage of resistance phenotypes was 100%. In 1967 Novick demonstrated the extrachromosomal linkage of the erythromycin resistance gene in Staphylococcus aureus (77). Since that time plasmid-encoded macrolide-lincosamide resistance has been described in several streptococcal species, Clostridium perfringens and Bacteroides fragilis (8,15,44,96,110). Weisblum and co-workers have demonstrated by DNA-DNA homology experiments at least in the case of Staphylococcus and Streptococcus the genetic determinant for macrolide-lincosamide resistance are closely homologous (108). This suggests a common evolutionary origin even though these two genera have not been demonstrated as exchangers of genetic information. It has been hypothesized that the natural producers of antibiotics probably represent the origin on the resistance genes involved (4). Weisblum has in fact found that in macrolide producing Streptomyces the 23 S rRNA is in fact methylated in a manner similar to that in erythromycin resistant

Staphylococcus(33).

The regulatory nature of the expression of macrolide-lincosamide resistance has been the subject of several publications. It was observed in staphylococci that resistance expression seemed to require exposure to erythromycin (106). In other words, only a small proportion of cells would form colonies on agar media containing high levels of erythromycin. On the other hand if the same cells had been growing in the presence of even very low concentrations of erythromycin (0.001 μg/ml) for only an hour, nearly the entire population of cells would survive to form colonies on media containing 100 μg/ml of erythromycin (106). This phenomenon has been termed erythromycin-inducible resistance (107). However, naturally occurring isolates of both staphylococci and streptococci have been found in which the expression of macrolide and lincosamide resistance is constitutive (78, 112). In this case, the efficiency with which the entire population of cells survives when challenged with erythromycin does not change whether or not there has been pre-exposure to erythromycin.

Novick has recently demonstrated that the macrolide-lincosamide resistance gene present on the *S. aureus* plasmid pI258 is part of a transposable DNA element called Tn 551 (79).

Tetracycline Resistance

Tetracycline is an inhibitor of protein synthesis. Its mode of action involves blocking the binding of aminoacyl t-RNA to the 30S ribosomal unit (94). The molecular basis for plasmid-encoded tetracycline resistance is not understood very well but some facts are known. It has been demonstrated that E. coli harboring this resistance fail to accumulate radioactively labeled tetracycline to the same extent as sensitive isogenic strains not harboring a tetracycline resistance plasmid (30,41). Secondly, in the enteric bacteria there is no suggestion that tetracycline resistance involves inactivation by structural alterations or alterations of the target site (58,60).

Plasmid-mediated tetracycline resistance has been described in many different genera of bacteria: Besides the enteric bacteria it has been found to occur in staphylococci, streptococci, clostridia and Bacteroides (8,44,55,83). The genetic homology of the tetracycline resistance determinant across wide genera differences (Staphylococcus versus E. coli) has not been approached experimentally. Within the enteric genera, Levy has identified at leas t a common membrane bound protein synthesized in the presance of various R plasmids which encode tetracycline resistance (58). Levy has called this the Tet protein since it is associated with induced tetracycline resistance (59).

The regulation and expression of plasmid-mediated tetracycline resistance has been most thoroughly studied in E. coli. T.J. Franklin initially demonstrated that pre-exposure to subinhibitory concentrations (0.5 µg/ml) of tetracycline for a short period of time resulted in an increased percentage of survival when cells were challenged with a high concentration of tetracycline [50 µg/ml](29). Levy and McMurry have shown that in conjunction with this behavior it is possible to detect in R plasmid containing minicells a specific membrane bound protein (tet protein) which is synthesized in response to the addition of subinhibitory amounts of tetracycline (59). It is Levy's hypothesis that the tet protein is involved in either prevention of tetracycline uptake or to enhancement of its export (113). This is based on two facts, first Franklin's initial observation that tetracycline does not accumulate within resistant cells to the same extent as sensitive cells (29). Secondly, the addition of oxidative phorphorylation inhibitors such as dinitrophenol cause normally plasmid-mediated tetracycline resistant cells to begin accumulating tetracycline (60). This suggests inhibition of an active transport system for tetracycline export. Although inducible plasmid-mediated tetracycline resistance seems to be the common occurrence among enteric bacteria, Franklin demonstrated that mutants which constitutively express tetracycline resistance can be readily isolated in the laboratory (31).

In some instances for the enteric bacteria, the tetracycline resistance determinants are contained within a transposon. For example, Kleckner et al have identified a tetracycline resistance transposon called Tn 10. Tn 10 has the ability to freely move from its resistance plasmid background (R-100) to either bacteriophage P22 or to the host chromosome in Salmonella or E. coli (51). However, in the case of the E. coli plasmid pSC101, the tetracycline resistance determinant does not appear to be a transposable gene sequence (personal communication D.J. Kopecko).

Bacteroides: General Considerations

The members of the Bacteroides genus are described as obligatory anaerobic, non-motile, non-spore forming, gram negative bacilli. They are found in association with warm blooded animals where they normally inhabit the oral cavity, intestinal tract and female genito-urinary tract. Extensive speciation of this phenotypically diverse genus has resulted in a rather bewildering array or organisms described in the literature. A monograph published by the Virginia Polytechnic Institute (VPI) is presently the best synopsis of Bacteroides taxonomy (39). The species which inhabit the human body can be divided into three groups based roughly on differing ecological niches and phenotypes. John Johnson has recently defined by use of DNA-DNA homology and phenotypic similarities the intestinal Bacteroides group as comprising a total

of seven named species and three additional unnamed species (46,47). The species of Johnson's grouping of intestinal Bacteroides are; B. fragilis, B. thetaiotamicron, B. ovatus, B. uniformis, B. vulgatus, B. distasonis, B. eggerthii and the three unnamed species designated B. fragilis subspecies a, "3452-A" and "T4-1". These ten species represent the first methodical taxonomic ordering for the Bacteroides genus. It should be taken into account that there are additional intestinal Bacteroides isolates which may deserve additional species ranking but these have yet to be thoroughly characterized from a taxonomic viewpoint (74). The second grouping of human Bacteroides species consists of those organisms more commonly found in the oral cavity than the lower alimentary canal. The more notable species in this group are B. melaninogenicus, B. oralis, B. orchraceus and B. corrodens. Lastly, the third grouping of Bacteroides based on ecological niche are those species more commonly isolated from the female genito-urinary tract. The species most often placed in this grouping are B. bivius, B. asaccharolyticus and B. disiens (39).

Little is known concerning the specific interplay of the intestinal Bacteroides with the human body. The facts we do know suggests that research on this goup of organisms will lead to a better understanding of human microbial ecology. Studies typlified by the Moore and Holdeman investigation of the normal fecal flora of twenty Japanese-

Hawaiians has shown that the intestinal Bacteroides group is far and away the predominating intestinal organism in terms of numbers and mass (74). It would be naive to suppose that the great preponderance of Bacteroides has come to reside in the human intestine under entirely neutral circumstances. Studies have shown that regimens of chemotherapy which lower the number of normal anaerobic intestinal inhabitants greatly increases the likelihood of infection and susceptibility to pathogens such as Salmonella and superinfections by staphylococci and pseudomonads (6). This suggests that the obligate anaerobic organisms in an ill-understood manner, out-compete pathogens and other organisms in the intestinal lumen and thus protects us from invasive bacterial disease.

Bacteroides fragilis: An Opportunistic Pathogen

The distinction between indigenous and pathogenic microflora is difficult to make. There are many examples of species of indigenous organisms which have extremely pathogenic potential when introduced into areas of the body which are devoid of host defenses or where the resistant microflora have been compromised by preoperative or cancer chemotherapy. The technological advances in the cultivation and identification of anaerobic bacteria from clinical materials have resulted in the recognition among infectious disease physicians that there are clearly virulent obligate anaerobic bacteria besides the well known

clostridial species (27,49,95). In this regard B. fragilis has come under scrutiny as an opportunistic pathogen (27,49, 95). Independent studies have shown it to be the most commonly isolated obligate anaerobic species in either pure or mixed infections (71,75,95). Very little information is available on its means of virulence or invasiveness although it appears that a polysaccharide capsule potentiates abscess formation in an intrabdominal rat model system. Additionally, the encapsulated strains of B. fragilis have been shown to adhere to rat peritoneal mesothilum better than unencapsulated strains of other intestinal Bacteroides species (82). There are several reports describing the production of extracellular enzymes associated with tissue destruction (e.g., hyaluronidase, DNase) by B. fragilis (5,87). A lipopolysaccharide is contained in the outer membrane of strains of B. fragilis (48). Structurally it lacks heptose and 2-keto-3 deoxyoctonate sugars which are characteristic of classic endotoxin molecules found in the faculatative anaerobic gram negative rods. Biologically the B. fragilis lipopolysaccharide also seems to lack many of the properties exhibited by classic endotoxin. It is not lethal for chick embryos nor does it induce local Schwartzman reactions in rabbit models even at inordinately high dose levels (48).

It does seem clear that B. fragilis must possess some factors resulting in an invasive capacity. B. fragilis ranks only twenty-seventh in prevalence of all the anaerobic bacteria identifiable in the feces (40). Yet B. fragilis ranks

as the number one anaerobic species found in infections involving anaerobes (74,95). If many infections were the direct result of contamination of wounds by fecal or intestinal matter one would then expect that the organisms most prevalent in that matter to be the most frequent causative agent. Clearly with the intestinal flora this is not the case, since anaerobic organisms such as B. vulgatus and Fusobacterium prausnitzii (ranked No. 1 and No. 2) are infrequently cultured from tissue infections (38,40).

Infections by B. fragilis present an unusual problem in chemotherapy since this organism is refractory to treatment with commonly employed penicillin, cephalosporin and aminoglycoside antibiotics. Clindamycin and chloramphenicol are antibiotics recommended for treatment of infections caused by B. fragilis (25). Unfortunately, these drugs are respectively associated with pseudomembraneous colitis and hematopoietic problems (99). The antiprotozoan drug metronidazole is effective against B. fragilis infections but its safety has been questioned (88,98). Clindamycin has become the drug of choice but reports of clinical isolates resistant to that drug have appeared in the literature (73, 83,89,96,110). If resistance to clindamycin were to become widespread in Bacteroides, a serious chemotherapy problem would ensue since there are few alternatives to clindamycin and those that are available (chloramphenicol and metronidazole) are unattractive due to possible dangerous sequela.

Bacteroides: Plasmids and Transferable Antibiotic Resistance

Once the importance of bacterial plasmids became established in the early 1970's, it became obvious to some that the resident microflora might serve as a repository of clinically important plasmids. The threat to treatment of future infections posed by widely disseminated resistance plasmids became apparent. The normal microflora also became a candidate for the origin of some of the antibiotic resistance genes. Members of the Bacteroides genus became likely candidates in this regard since they are a rich part of the human microflora and they are inheritantly resistant to many antibiotics.

The first publication on the isolation of plasmid DNA from any Bacteroides species appeared in 1974 (92). Stiffler et al examined four different clinical isolates of B. fragilis and found plasmid DNA in three out of the four strains. First they purified the DNA by CsCl-EB dye-bouyant density-equilibrium ultracentrifugation. They then characterized the size of the plasmids by sucrose gradient centrifugation and electron microscopy. They found two strains to harbor a 4 Mdal plasmid and the third strain to possess two plasmid species 2.7 and 16 Mdal in size. These workers were unable to correlate the presence of plasmid DNA with any phenotypic property including antibiotic resistance.

Next came two reports on the isolation of plasmid DNA in oral Bacteroides species. Damle and Sved found a 17

Mdal plasmid in a strain of B. melaninogenicus and a 5 Mdal plasmid in B. ochraceus (20). In both strains, the authors were unable to ascribe a phenotype to the plasmids. Guiney and Davis found in a strain of B. ochraceus (2228) two plasmid species 25 and 70 Mdal in size (35). At the time of this initial report, these workers were unable to attribute a phenotype to these plasmids. However, in a publication which will be described later in this review, they eventually were able to assign several antibiotic resistances to the larger plasmid species.

In 1976 Tinnell and Macrina published a survey of the presence of plasmid DNA in the intestinal Bacteroides group (101). This survey was performed in order to provide a better idea as to whether or not the intestinal Bacteroides represented a sizeable plasmid reservoir. They examined a strain from each of Johnson's nine DNA-DNA homology groups (the groups given species name) for the presence of plasmid DNA using the techniques of CsCl-EB dye-buoyant density ultracentrifugation and sucrose gradient analysis. Four of the nine strains gave satellite bands in CsCl-EB gradients suggesting the presence of CCC DNA. From sucrose gradient analysis they determined the molecular size of the plasmids and found them to vary in size from 3 to 31 Mdal with no multiple plasmid containing strains being evident. Again no phenotypes were accorded and these plasmids remain of cryptic nature.

To date the most thorough analysis of the plasmid content in the intestinal Bacteroides has been performed by Mays and Johnson of the VPI Anaerobe Laboratory. As part of his doctoral thesis work, Dr. Mays examined 120 strains of Bacteroides for plasmids (67). He employed the Currier and Nestor technique for plasmid isolation and used agarose gel electrophoresis for size characterization (18,68). It was found that 43 percent of the strains examined had plasmid DNA and plasmids were present in isolates of each of Johnson's DNA-DNA homology groups. This work best indicates that the intestinal Bacteroides group is a rich reservoir of extrachromosomal DNA. A summary of Mays' data is presented on Table 3. Mays employed a computer analysis in order to see if any of 81 different phenotypic traits could be assigned to any of four different reference plasmid containing strains. No correlation was seen and it was concluded that all of the plasmids found were cryptic.

Mays also carried out plasmid-plasmid DNA-DNA homology studies. A very interesting finding was a small 3 Mdal plasmid (T1-1) possessed moderate (30-60%) or high (61-100%) homology with plasmid DNA found in 34 out of 52 plasmid containing strains. This suggests the wide dissemination of a common extrachromosomal sequence. The means of this dissemination and the nature of the genetic information encoded by this molecule is a speculative matter.

Initial attempts at demonstrating transferable antibiot-

Table 3

Distribution and Size of Plasmid DNA Found in 120 Strains of *Bacteroides* (67)

DNA-DNA homology group	Number of strains examined.	Number of strains with plasmid DNA	Plasmid size (Mdal)	Number of Multiple plasmid containing strains
B. fragilis I	16	7	2 - 59	2
B. fragilis II	3	1	22	0
B. thetaiotaomicron	5	3	2 - 23	2
B. ovatus	11	3	2 - 23	3
"3452-A"	12	8	2 - 40	3
B. uniformis	26	11	2 - 53	5
B. eggerthii	6	2	3 - 7	2
B. fragilis subs.A	6	1	3	0
B. vulgatus	14	7	2 - 65	4
B. distasonis	11	7	2 - 60	6
"T4 - 1"	9	1	62	0
No designated group T1-42	1	1	2,5	1

ic resistance from resistant Bacteroides to other sensitive bacteria were not successful (1,23). There are three reports of intergeneric transfer of antibiotic resistance between intestinal Bacteroides and E. coli but in each instance there is a failure to physically demonstrate a plasmid in the donor or in recipients upon inheritance of the antibiotic resistances (10,65,114). The transfer of β-lactam, tetracycline and chloramphenicol resistance from B. fragilis to E. coli has been claimed by Mancini and Behme, as has the transfer of ampicillin resistance from E. coli to Bacteroides by Burt and Woods (10,65). Young and Mayer claim to have transformed E. coli to ampicillin resistance using a small Bacteroides plasmid (114). As of yet they have failed to demonstrate the stable-maintenance of this plasmid and the drug resistance in E. coli. Additionally, they describe the "conjugational" transfer of ampicillin resistance from resistant B. fragilis strains to E. coli but again the resistance phenotype appears to be unstable in the recipients. The unequivocal demonstration of plasmid-mediated intergeneric transfer of antibiotic resistance has been shown by Guiney and Davis (36). They demonstrated that B. ochraceus strain 2228 which is resistant to kanamycin, chloramphenicol and tetracycline could transfer those resistances to an E. coli strain in a manner suggestive of bacterial conjugation. Associated with the transfer of the three drug resistances was the transfer and maintenance of a 70 Mdal plasmid species in the E. coli recipient.

In 1979, reports of tranferable clindamycin-erythromycin resistance in B. fragilis appeared from the laboratories of Talley, Sebald and Macrina (83,96,110).

In Talley's case, B. fragilis strain TMP 10 which is resistant to clindamycin and erythromycin could tranfer those resistances to a drug sensitive strain of B. fragilis using the filter mating technique (96). The drug resistant progeny in this mating could in turn serve as donors of clindamycin-erythromycin resistance to a sensitive strain of B. thetaiotaomicron. Thus, Talley demonstrated an apparent maintenance of conjugal proficiency in the progeny upon inheritance of the drug resistances. When the plasmid content of TMP 10 was examined, it was found to harbor several plasmid species varying in size from 2 to over 20 Mdal. When the drug resistant progeny were examined for plasmid content, they were found to bear different combinations of the plasmid species seen in the donor. However, it appeared that two plasmids were commonly found in all transfer proficient, drug resistant progeny (a 2 and a 20 Mdal species). A spontaneously occurring clindamycin-erythromycin sensitive variant of one of the drug resistant progeny was seen to have undergone the loss of the 2 Mdal plasmid. From these results, Talley et al concluded that the 20 Mdal plasmid conferred the transmissability of resistance and that the 2 Mdal plasmid bore the resistance gene(s). Recently there has been a reevaluation of these results. In abstracts for the 19th Interscience Conference on Antimicrobial Agents

and Chemotherapy, Talley et al now report that neither the 2 nor the 20 Mdal plasmid is involved, instead a single 10 Mdal plasmid pBF104 confers both clindamycin-erythromycin resistance and transmissability (97). In the same abstract Talley reports for the first time experiments designed to elucidate the mechanism of the genetic transfer event. Close cell to cell contact is required between TMP 10 and any recipient strains for transfer to occur. Additionally the transfer is insensitive to DNase treatment. From these results they've concluded that conjugation is the most likely genetic event for the resistance transfer in TMP 10.

Sebald's group has reported that two different strains of Bacteroides (BEN and 92) resistant to clindamycin, erythromycin and virginiamycin (a streptogramin B antibiotic) can transfer those resistances en bloc to a sensitive strain of B. fragilis (84). Their transfer protocol involves growing the donor and recipient cells in mixed culture on the surface of a nutrient agar media. The BEN strain is a B. distasonis which is tetracycline resistant and capable of also transferring that resistance. In their original publication, strain 92 is described as being tetracycline resistant but incapable of transferring that resistance. They reported that both strains could be observed to undergo spontaneous loss of the clindamycin-erythromycin resistance but not the tetracycline resistance. The frequency at which the spontaneous loss of clindamycin-erythromycin resistance occurred among colonies scored for drug

resistance was one percent. From this evidence they concluded that the clindamycin-erythromycin resistance gene(s) were likely to be of an extrachromosomal nature. They did not provide any physical evidence for plasmid-mediated drug resistance transfer with either strain. Additionally no experiments were described which would identify what type of genetic event was responsible for the resistance transfer.

In a second publication, this same research group describes for strain 92 what is apparently a "tetracycline-inducible" mechanism for the transfer of tetracycline resistance (84). When strain 92 is grown overnight in the presence of 5 μg/ml tetracycline, the strain then becomes proficient in the transfer of tetracycline resistance. The inducibility of the tetracycline resistance transfer was found to be reversible. When a transfer proficient strain was shifted to tetracycline-free media it lost the ability to transfer tetracycline resistance after several overnight subcultures. After twenty sequential overnight subcultures in the presence of 5μg/ml tetracycline, they found that a strain of 92 had become constitutive for transfer of tetracycline resistance. In this instance, the inducitility of transfer was not reversible. Further, they provided evidence that the expression of tetracycline resistance in strain 92 was inducible by exposure to subinhibitory amounts of tetracycline. Two overnight subcultures of strain 92 were made, one grown in the presence of 0.5 μg/ml tetracy-

cline and the other grown in the basence of tetracycline. Growth of the two cultures then was monitored optically. The culture inoculated with the tetracycline containing overnight culture soon began to grow in a logarithmic fashion with virtually no lag phase. The other culture which had not been pre-exposed to tetracycline began logarithmic growth only after a lag phase of three hours.

The publications on transferable clindamycin-erythromycin resistance orginating from Macrina's laboratory involve Sebald's strain 92 (109,110). This thesis is in fact based upon the results of experiments performed with strain 92 (V479 in this laboratory). The description of these results can be found in the following text.

MATERIAL AND METHODS

Bacterial Strains

The principal bacterial strains employed in this study along with their laboratory designation and source are listed on Table 4. The designation and source of additional strains resulting from matings and deletion studies will be described as they occur in the results section.

Chemicals

The source and handling of all chemicals employed in this study will be described as their use occurs in the materials and methods.

Antibiotics

All antibiotics with the exception of ampicillin were made as solutions and filter sterilized (0.45 micron filter, Millipore Corp.).

Clindamycin HCl and lincomycin HCl were provided courtesy of the Upjohn Company. Both drugs were made as 10 µg/ml aqueous stock solutions and stored at 4^{o} C.

Erythromycin (Sigma) was made as a 50 µg/ml ethanol solution and then diluted 1 to 1 with distilled H_2O for a stock solution of 25 µg/ml.

Rifampicin (Sigma)was made as a 1 µg/ml 10% methanol solution and used promptly in all media.

Table 4

Principle *Bacteroides* strains employed in this study

Strain Designation	*Bacteroides* species	Source or deriviation
V479	*fragilis*	M. Sebald (strain 92)
V479-1	*fragilis*	This laboratory
V514	*fragilis*	This laboratory
V218	*uniformis*	P. Hylemon (strain 0061-1)
V531	*fragilis*	T. Wilkins (strain B-70)

Na ampicillin was given courtesy of Bristol Laboratories. It was directly added as powder to cooled autoclaved media and carefully monitored for contamination.

Tetracycline HCl (Lederle and Sigma) was made as a fresh 1 μg/ml aqueous solution and used promptly in all media.

Buffers

TE: 50 mM Tris (hydroxymethyl) aminomethane [Tris base, Sigma] and 5mM disodium ethylenediaminetetraacetate [EDTA, Sigma]. The pH was brought to 8.0 with concentrated HCl.

TES: Same as for TE with addition of 50 mM NaCl (Baker).

TB: 89 mM Tris (hydroxymethyl) aminomethane [Tris base], 89 mM boric acid [Sigma] and 2.5 mM disodium ethylenediaminetetraacetate [EDTA]. The pH brought to 8.0-8.2 with HCl.

Enzymes

The source and preparation of commercial enzymes used in preparative techniques will be described in the text as their use arises.

All restriction endonucleases used in plasmid characterization with the exception of Bam HI (Miles Laboratories, Elkhart, Indiana) were obtained from Bethesda Research Laboratories (BRL, Rockville, Md.). The enzymes were stored at -20°C as suggested by the manufacturers. Assay

buffers for the restriction endonucleases were made as 10-fold concentratedsolutions of the buffers recommended in the BRL information bulletin accompanying each enzyme. Complete and appropriate digestion of DNA was monitored by subjecting bacteriophage lambda DNA (BRL) to each restriction enzyme. The digest products were analyzed by agarose gel electrophoresis.

Media and Maintenance of Stock Cultures

A single complex growth medium (PHC) was employed in growing large cultures of Bacteroides. This medium consisted of 17.6 g/ℓ Antibiotic medium No. 3 (Difco) supplemented with 1g of cysteine HCl/ℓ (sigma), 1g D-glucose/ℓ (Difco) and 10 ml of a Vitamin K-heme solution/ℓ (39). The pH was adjusted to 7.0 by the addition of 1N NaOH.

A minimal medium developed by D. Callihan and F.E. Young called Bacteroides Genetic Medium (BGM) was employed in several mating experiments. The BGM formulation is; glucose 0.5 g/ℓ, hemin 0.5 µg/ℓ, cysteine HCl 0.12g/ℓ, L-methionine 0.018 g/ℓ, $CaCl_2 \cdot 2H_2O$ 0.5 µg/ℓ, KH_2PO_4 1.05g/ℓ, K_2HPO_4 0.9 g/ℓ NaCl 0.52 g/ℓ, $(NH_4)_2SO_4$ 0.045 g/ℓ and $MgCl_2 \cdot 6H_2O$ 0.045 g/ℓ (D. Callihan personal communication). The last five salts were made as a 10X minimal salts solution.

Whenever a solid agar medium was required agar (Difco) at a final concentration of 1.5 percent was added. All solid media was usually prepared on the day it was to be used. Stoppered liquid media was stored for periods of up

to ten days.

Stock cultures were maintained in 10 ml of chopped meat medium and stored at room temperature under 10 percent carbon dioxide and 90 percent nitrogen as described in the VPI Anaerobe Manual (39). Transfers were made approximately every two months. Strains displaying tetracycline resistance (e.g., V514) were kept in a stock culture containing 1 µg/ml tetracycline to insure the maintenance of tetracycline resistance. Clindamycin was not put into the stock cultures since the cultures did not appear to lose that resistance readily. Freezer stocks of Bacteroides were made by suspending a heavy lawn of cells grown on solid PHC in a 30 percent glycerol-PHC broth solution. The cell suspension was divided into two aliquots, one stored at -20°C and the other at -70°C.

Culture Techniques

Culture plates were incubated anaerobically using the Gas-Pak system (BBL, Cockeysville, Md.). Liquid tube and flask cultures were incubated under an oxygen-free, 10 percent carbon dioxide and 90 percent nitrogen atmosphere delivered by an anaerobic gas flushing system designed by the VPI Anaerobe Laboratory (VPI Anaerobe Culture System, Bellco, Vineland, N.J.).

Antibiotic Sensitivity Testing

The minimal inhibitory concentration (MIC) for an anti-

biotic was determined by an agar dilution technique (63). Overnight PHC broth cultures were diluted in phosphate buffered saline to yield an inoculum size of approximately 10^3 colony forming units per agar plate. The inoculum was spread onto PHC agar plates containing various stepwise concentrations of an antibiotic. The MIC was judged to be the highest antibiotic concentration where the inoculum yielded 100% recovery of resistant colonies versus an antibiotic free control plate. This technique yielded reproducible results.

Rifampicin Resistant Mutant Isolation

The following protocol was developed in order to isolate spontaneously occurring rifampicin resistant mutants of various Bacteroides strains. An overnight PHC broth culture was diluted ten fold into a PHC broth containing 0.1 µg/ml rifampicin. This then was incubated until turbid (24-72 hours) at 37°C. Once turbid, this culture was used to inoculate a 0.5 µg/ml rifampicin PHC broth. This stepwise subculture process was carried out at 1 µg/ml and finally 10 µg/ml rifampicin. Once a strain had grown to turbidity at 10 µg/ml, it was streaked for single colony isolation onto PHC agar plates containing 10 µg/ml rifampicin. A single colony on this plate then was stocked cultured in chopped meat broth.

Mating Procedure

Stock cultures of each donor and recipient strain were used to inoculate 10 ml PHC broth cultures and these broths were incubated overnight at 37°C. Each PHC broth then was diluted 10-fold and allowed to grow to mid-to-late exponential phase. Growth was monitored with a Spectronic 20 (Bausch and Lamb, Rochester, N.Y.) and cells were generally collected at an absorbance of 0.5 to 0.6 corresponding to ~$1x10^8$ cells/µl. One ml of recipient and 0.5 ml of donor then were placed in a sterile 1.5 ml polypropylene Eppendorf centrifuge tube (AGonics, Brooklyn, N.Y.). The cell mixture was pelleted by a two minute centrifugation at room temperature in a Eppendorf centrifuge model 5412. Nearly all of the supernatant then was drawn off with a sterile pasteur pipette leaving the cell pellet and approximately 0.2 ml of supernatant. The cell pellet then was resuspended in the remaining supernatant. The resuspension was pipetted onto a sterile membrane filter (type HA, 25 mm diameter, 0.45 µm pore size; Millipore Corp.) which had been placed onto freshly prepared antibiotic-free PHC agar plate. The filters and plates then were incubated anaerobically in Gas-Pak jars for 24 hours (unless otherwise stated). Following this incubation, the filters were transferred to a sterile 40 ml screwcap, Nalgene centrifuge tube. One ml of sterile phosphate-buffered physiological saline was added and the tube was agitated vigorously on a Vortex mixer for

approximately 30 seconds. Appropriate dilutions of the cell suspension were then plated on PHC agar supplemented with the appropriate antibiotics. Antibiotics were employed at the following concentrations unless noted otherwise; rifampicin 10 µg/ml, lindamycin, clincomycin and erythromycin 5µg/ml, tetracycline 10 µg/ml and ampicillin 1 µg/ml. Occasionally the BGM medium was substituted for the PHC medium in progeny selection. When BGM was employed, rifampicin was not utilized to counterselect against the donor. Instead a carbohydrate (e.g. arabinose) which the recipient but not the donor could utilize for growth was substituted for glucose in the BGM formulation . Drug resistant progeny (transcipients) were reisolated twice by streaking well isolated single colonies on the medium employed in the initial selection. Once the subculturing process was completed, the new transcipients were stocked and examined for unselected phenotypes and plasmid content.

Plasmid DNA Preparation: Mini-lysate Technique

A fast, simple plasmid isolation procedure for Bacteroides strains was developed by scaling down what is essentially the SDS-high NaCl technique of Guerry et al (34). The mini-lysate technique results in plasmid preparations relatively high in contamination with linear chromosomal fragments thereby preventing the use of this technique for plasmid preparations suitable for electron microscopy and restriction endonuclease digestion. However, this technique

was ideally suited for quick screening of newly acquired Bacteroides strains and trancipients for plasmid content.

A chopped meat stock culture was used to inoculate a 10 ml PHC broth culture which then was grown overnight at 37°C. Depending upon the cell yield anywhere from 6 to 10 mls of the PHC broth culture was harvested by centrifugation (SS34 rotor, 8,000 rpm, 10 minutes, 4°C), the supernatant discarded and the cell pellet transferred to a 1.5 ml polypropylene Eppendorf centrifuge tube. The pellet then was resuspended in approximately 1.2 ml of 1X TES buffer and repelleted in a table model Eppendorf centrifuge (~4 minutes, room temperature). The supernatant was discarded and the washed cell pellet resuspended in 0.25 ml of a 25% sucrose solution (0.05 M Tris, pH8). Added to the cell suspension was 6 µl of RNase [5 mg/ml in 0.25 M Tris, pH8] (Sigma), 13 µl EDTA [0.25M] and 17 µl of egg white lysozyme [10 mg/ml in 0.25 M Tris, pH8] (Sigma). This mixture was then incubated for 10-15 minutes at room temperature. Next, 20 µl of 20 percent SDS [0.01 M Tris, pH8] (Pierce Chem., Rockford, Ill.) was added and incubated at room temperature until visable cell lysis had occurred (clearing). 50 µl of a 5M NaCl solution then was added and the lysate incubated at 4°C for at least three hours. Following the SDS-high salt precipitation, the chromosomal DNA and cellular debris was separated from the plasmid DNA by centrifugation at 12,000 rpm for 20 minutes at 4°C. The pelleting was performed in a Sorvall RC5 refrigerated preparative centrifuge

using a SS-34 rotor fitted with adapters for 1.5 ml Eppendorf centrifuge tubes. The pellet was gently removed and discarded using a pasteur pipette. 0.3 ml of freshly redistilled phenol (saturated with 1 X TES) was added to the supernatant and the mixture was briefly vortexed. Next, 0.3 ml chloroform:isoamyl alcohol (24:1) was added and the mixture was again vortexed. Phase separation then was achieved by centrifugation of the 1.5 ml tube in the Eppendorf centrifuge for one minute. The top aqueous layer (~0.5 ml) was removed with a pasteur pipette and placed into a new 1.5 ml Eppendorf centrifuge tube. The tube then was filled with ethanol (20°C, ~2 volumes) and 13µl of 5M NaCl added. The tube then was kept at -70°C for at least one hour and the ethanol-precipatated DNA then was pelleted by centrifugation at 7,000 rpm, -20°C for 20 minutes. The ethanol was poured off and the Eppendorf tube was kept inverted until all the ethanol had evaporated. The precipatated DNA then was brought into solution by the addition of 60µl of sterile deionized water. Approximately 15 µl of plasmid DNA prepared in this manner was in sufficient concentration to enable easy visualization of plasmid DNA in agarose gels stained with ethidium bromide.

Purification of Plasmid DNA

The following procedure was employed to acquire large amounts of concentrated plasmid DNA sufficiently free of chromosomal contamination for use in electron microscopy

and restriction endonuclease digestion. A 300 ml PHC broth culture of late log phase cells was harvested by pelleting in a Sorvall RC5 refrigerated centrifuge (8,000 rpm, 4°C., 15 minutes) using a GS-3 rotor. The supernatant was discarded and the pellet resuspended in 50 mls TES buffer. The cell suspension was repelleted as before and again the supernatant was discarded. The TES washed cell pellet then was resuspended in 10 ml of 25% sucrose (0.05 M Tris, pH8) and the suspension split into five 2 ml aliquots. To each aliquot 0.5 ml of freshly prepared 5 mg/ml lysozyme (0.25 M Tris, pH8 was added and the lysozyme-cell suspension incubated for 15 minutes at room temperature. Next 0.5 ml of 0.25 M EDTA (pH8) along with 0.25 ml of 1 mg/ml RNase (0.25 M Tris, pH8) was added and the entire mixture kept for 10-15 minutes at room temperature. To achieve cellular lysis, 0.16 ml of 20% SDS (0.02 M Tris, pH8) was added and incubated at room temperature for 15 minutes to 30 minutes. Once cellular lysis had become evident, 0.85 ml of 5M NaCl was added and the preparation kept at 4°C. for at least 3 hours. On some occasions the detergents Brij 58 (ICI, Wilmington, Del.) or Triton X-100 (New England Nuclear, Boston, Mass.) were used instead of the SDS-high NaCl. In the case of Brij 58, 3.2 mls. of a 2% Brij 58, 2% DOC (0.05M EDTA, 0.05M Tris) was added to each aliquot. For Triton X-100, 3.2 mls. of a 1% Triton X-100 (0.05M EDTA, 0.05M Tris, pH8) was added to achieve lysis. The cellular debris and chromosomal DNA was separated from the plasmid

enriched supernatant by centrifugation of the preparations in the RC5 centrifuge using the SS34 rotor at 20,000 rpm, 20 minutes -20°C. (for the SDS-NaCl preparations) or 4°C (for the Brij 58 and Triton X-100 preparations).

The supernatant acquired following the clearing spin then was subjected to cesium chloride-ethidium bromide dye bouyant density ultracentrifugation patterned on the technique developed by Vinograd and associates for purification of circular virus DNA molecules (95). The method developed for Bacteroides plasmid DNA involved mixing 6.0 mls of cleared lysate (the supernatant), 7.7 g cesium chloride (Kawecki-Berylco, Reading, Pa.) and 0.57 ml of 10 mg/ml ethidium bromide. This CsCl·EB·DNA solution then was poured into 10 ml. polypropylene tubes (Beckman) and paraffin oil (Baker) was layered on top. The tubes then were sealed with Beckman cap assemblies and the tubes placed in a Beckman fixed angle 50 Ti roter. Equilibrium ultracentrifugation then was achieved by subjecting the preparations to 40,000 RPM at 18-20°C for 48-72 hours in either a Beckman L2-65 or L5-50 preparative ultracentrifuge. Following ultracentrifugation, the plasmid DNA present in such gradients was visualized by illuminating the gradient tube with a long-wave ultraviolet light source (Ultra-Violet Products, San Gabriel, California). The lower flourescent band corresponding to CCC DNA (plasmid DNA) then was removed by needle and syringe aspiration from the side of the tube. Ethidium bromide was removed by

extraction with chloroform:isoamyl alcohol (24:1) and the cesium chloride effectively removed by dialysis against at least 8ℓ of 1 X TE buffer. The DNA concentration was measured spectrophotometrically using either a Zeiss M4 QIII or Beckman model 35 spectrophotometer. One O.D. unit at 260 nm was taken to represent a DNA concentration of 50 µg/ml (64). Purified plasmid DNA preparations were stored at $4^{o}C$ in 2 ml screw cap glass vials.

Agarose Gel Electrophoresis

Identification and approximate size determinations of plasmid DNA prepared either by the mini-lysate or CsCl-EB technique were performed by the agarose slab gel electrophoresis technique of Meyers et al (68). Agarose slab gel electrophoresis was also used to estimate the sizes of linear DNA fragments following digestion of plasmid DNA with restriction endonucleases (7).

Agarose (Seachem, Rockland, Me. or BRL, Rockville, Md.) in 1 X TB buffer at concentrations varying from 0.6% to 0.8% depending on the experiment was cast into slab gels by pouring the molten agarose between two clean glass plates separated with lucite spacers. Immediately after pouring the agarose, a lucite comb was inserted into the top of the gel creating sample wells. The final slab gel dimensions were 15 X 10 X 0.38 cm. Once the gel had solidified, the comb was removed and the gel was clamped into a vertical slab gel electrophoresis unit (Savant, Long Island City, N.Y.).

Approximately 600 mls of 1 X TB running buffer then was split between the top and bottom buffer chambers. DNA mixed with one quarter volume of a bromphenol blue tracking dye solution (0.07 mg/ml bromphenol blue [Sigma] and 0.07 g/ml SDS in a 33% glycerol aqueous solution) was put into the sample wells with a 100 µl capillary pipette. In all cases between 0.5 µg and 2.0 µg of DNA was applied to each well. Power was supplied with a Heathkit regulated high voltage power supply, Model SP2717. Electrophoresis was usually performed at 40 mA (~120V) until the blue tracking dye had migrated into the bottom supporting sponge (~ 2 hours). The running buffer then was poured into a glass baking dish and ethidium bromide added to achieve a final concentration of approximately 3 µg/ml. The gel was stained in the solution 10-15 minutes and then destained in a running water bath for approximately one-half hour. The gel then was illuminated on a short-wave ultraviolet transilluminator (Model C-61, Ultra-Violet Products Inc., San Gabriel, California) and photographed with a Polaroid MP4 Land Camera fitted with a Wratten No. 9 filter. The film used was a 4 x 5 positive/negative black and white Polaroid Type 55 Instant Land Film.

The positive photographic image then was used to measure the relative migration distance of the unknown plasmids or linear DNA fragments versus an included known standard reference of the appropriate DNA physical form. The migration behavior of CCC DNA can only be compared to

the behavior of other CCC species and by analogy the same is true for linear DNA species. Molecular size estimates of unknown CCC DNA or linear DNA species were made by comparing the logarithm of their migration distances to a standard curve of the logarithm of the molecular weight of known DNA species versus the logarithm of the respective migration distances for the known DNA's [the two factors are inversely related] (68). The reference standards for CCC DNA were the 8 plasmids present in a single strain of E. coli, V517 (62). Plasmid DNA from V517 purified by CsCl-EB ultracentrifugation was run on every gel where size estimates of unknown plasmids were made. The plasmids in V517 are 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8 and 1.4 Mdal in size. The linear DNA standard employed in restriction endonuclease digests was either Hind III or EcoRI digested bacteriophage lambda DNA. The sizes of these fragments are (Hind III) 15, 6.4, 4.3, 2.9, 1.6 and 1.4 and (EcoRI) 13.7, 4.5, 3.5, 3.0 and 2.3 (BRL product information). The migration distances were measured from the edge of the sample well to the leading edge of the DNA band. A draftsman's divider was used to span the migration distance and this span was measured on an engineers ruler (1/60th in scale). The logarithms of migration distance for the known DNA species were entered with the logarithm of their known molecular size into a linear regression program provided by a Texas Instruments programmable calculator (Models TI58 and TI59 were both used). Once the calcula-

tor generated a standard curve, the logarithm of the migration distance of an unknown DNA species was entered into the program and an estimate of its molecular size was provided.

The photographic negatives of gel pictures containing plasmids digested with restriction endonucleases were examined by densitometry. A Corning 750 scanning densitometer (wavelength setting 550 nm) printed densitometric tracings of the plasmid digests and calculated the areas of peaks corresponding to each DNA fragment. A ratio then was made for each peak area to the total of peak areas. This ratio was compared to the ratio of the respective fragment size to total plasmid size. Hence, a fragment was judged to be a doublet species when its representative peak area appeared to be twice the proportion predicted by the size ratio.

Partial digest fragments were those faint bands appearing in the pictures of restriction endonuclease digests carried out under limiting conditions. The incomplete digests were accomplished by diluting the restriction endonuclease, brief digestion periods (~ 5 minutes) or digestions at $0^{o}C$.

Isolation of Clindamycin-Erythromycin Sensitive Variants of Resistant Strains: Spontaneous Occurring Sensitive Strains

Chopped meat stock cultures of clindamycin-erythromycin resistant strains were used to inoculate a *PHC* broth which was grown overnight at $37^{o}C$. The overnight

culture then was diluted and plated onto plain PHC agar so as to yield approximately 150-200 colonies per plate. The plates were incubated 48 hours at 37°C until large-well isolated colonies appeared. The colonies then were individually touched with a sterile wooden toothpick and this was used to inoculate a plain PHC agar plate and a PHC plate containing 5μg/ml clindamycin and/or erythromycin. In this manner 100 colonies were tested per set of plates. Any sensitive clones were stocked and then tested for several key phenotypes (indole, catalase and obligate anaerobic growth). Appropriate clones then were screened for plasmid content by the mini-lysate technique and agarose gel electrophoresis.

Isolation of Drug Sensitive Variants Following Growth in the Presence of Ethidium Bromide

Overnight PHC broth cultures of clindamycin-erythromycin resistant strains were used to inoculate PHC broths containing ethidium bromide (Sigma). Three different ethidium bromide concentrations were usually employed: 0.5, 0.05 and 0.005 mM. The ethidium bromide PHC broth cultures then were incubated at 37°C until turbid (48-72 hours). Sensitive clones following this treatment were isolated in the manner previously detailed.

Reversion to Clindamycin-Erythromycin Resistance

Clindamycin-erythromycin sensitive clones isolated by the previous procedures were tested for spontaneous reversion to the clindamycin-erythromycin resistance phenotype

in the following manner. Plain PHC broths were inoculated from stock cultures and incubated overnight at 37°C. The following day 0.1 ml of this culture was spread onto PHC agar plates containing 5 µg/ml clindamycin and/or erythromycin. At the same time a viable count of the cells in the overnight culture was made by plating appropriate dilutions onto PHC plates. After 72 hours of incubation at 37°C , all plates were examined for clindamycin-erythromycin resistant revertants and the viable count of the overnight broth culture was determined.

The sensitive clones were also tested to see if various chemical mutagens could cause a reversion to clindamycin-erythromycin resistance. The technique employed in this investigation was essentially that of Balbinder as modified and described in Miller's Experiments in Molecular Genetics (3,70). Filter paper discs (~ 1 cm diameter) were soaked in one of the following chemical solutions: 10 mg/ml acridine orange (Calbiochem), 10 Mg/ml ethidium bromide, ethyl methyl sulfonate (Eastman), 1 M hydroxylamine HCl [pH6] (Fisher Scientific) and 2 mg/ml nitrosoguanidine (Aldrich, Milwaukee). The mutagen soaked disc then was placed in the center of a PHC agar plate containing 5 µg/ml clindamycin. This plate had just been seeded with 0.1 ml of an overnight PHC culture of a drug sensitive clone. These plates then were incubated and examined periodically for up to six days for colony formation.

Plasmid Copy Number Determination

Chopped meat stock cultures were used to inoculate ~6 ml of PHC broth containing 50 μCi/ml ^{3}H-thymidine and the culture then was permitted to grow until mid-log phase. The cells then were harvested and washed twice with 10 ml 1 X TES buffer. After the second wash, the cell pellet was resuspended in 0.4 ml of a 25% sucrose [0.05 M Tris, pH8], 1 mg/ml RNase, 1 mg/ml lysozyme mixture. The suspension was incubated 30 minutes at 37°C then placed on ice for one minute. Next, 0.2 ml of 2% Sarkosyl NL 97 [1 X TES] (Geigy Chem.) was added and the mixture incubated on ice for 30 minutes. 0.6ml 1 X TES buffer then was added and the lysate sheared by drawing it in and out of a 10 ml pipet 20 times. 0.5 ml of the lysate then was subjected to CsCl-EB dye buoyant density ultracentrifugation by centrifugation at 40,000 RPM, 20°C for 48 hours. Following centrifugation the gradients were punctured from the bottom and 7 drop fractions collected in 13 X 100 mm glass tubes. 50 μl of each fraction was placed onto Whatman 3 mm discs and the DNA precipitated by two washes in ice cold 5 percent TCA followed by two washes in 95 percent ethanol. Each disc then was placed in 1 dram glass vials and 3 ml of 0.8 percent 2.5-bis-[2-(5 tert-butylbenzoxazolyl]thiophene in toulene added. Scintillation counting of each fraction was performed using a Beckman LS-350 liquid Scintillation System.

Electron Microscopy: Contour Length Measurements

The molecular weight of an unknown plasmid species was

determined by measuring the contour lengths of their open circular forms in electron micrographs. Those lengths then were compared to the length of a known DNA species included in the same microscopy preparation. The method of preparing DNA molecules for electron microscopy was essentially that of Kleinschmidt as modified by Kopecko et al (52,53).

Purified plasmid DNA in 1 X TE buffer was converted from predominantly the CCC form to the open circular form by one of three different methods: prolonged storage at $4^{o}C$, heating at $75^{o}C$ for 10-15 minutes and timed exposure to gamma irradiation in a ^{60}Co irradiator (Gammacell 220). The irradiation method was performed by Dr. Dennis J. Kopecko using a radiation source available at the Department of Bacterial Immunology, Walter Reed Army Research Institute. 0.1 to 0.2 µg of DNA was made into a hyperphase by adding ammonium acetate (final concentration 0.5 M). The DNA hyperphase was spread onto a 0.25 M ammonium acetate hypophase by gently pipetting the hyperphase just above the interface on a clean glass slide half-immersed into the hypophase. The hyperphase was permitted to spread across the hypophase and stabilize for approximately 7 minutes. Parlodiom (E.F. Fullam, Schenectady, N.Y.) coated copper grids (300 mesh, E.F. Fullam) then were used to pick up the DNA resting on the hypophase. The DNA then was stained in 0.05 mM uranyl acetate (Fisher Scientific) in 95 percent ethanol for 30 seconds and then washed 30 seconds in 95 percent ethanol. The grids were rotary-shadowed with platinum-pallidium (80:20, Ted Pella Inc., Tustin, Ca.) in a Denton Vacuum

DV-515 shadower. The grids then were scanned in an Hitachi HS-7s electron microscope at magnifications varying from 6,750 to 9,740 X. Appropriate molecules were photographed with Kodak Electron Image Film 4463. Contour lengths then were measured either by enlarging the negatives with a Simmons-Omega 4 X 5 D-2 enlarger and tracing the molecular images with a map measuring device or by use of a Numonics projection device integrated with a Numonics electronic graphics calculator. The open circular forms of pSC101 [6.02 Mdal] or pSC 185 [9.2 Mdal] or pBR 322 [2.9 Mdal] were used as internal reference standards (14,54,93).

RESULTS

Phenotypic Characterization and Antibiotic Sensitivity Testing

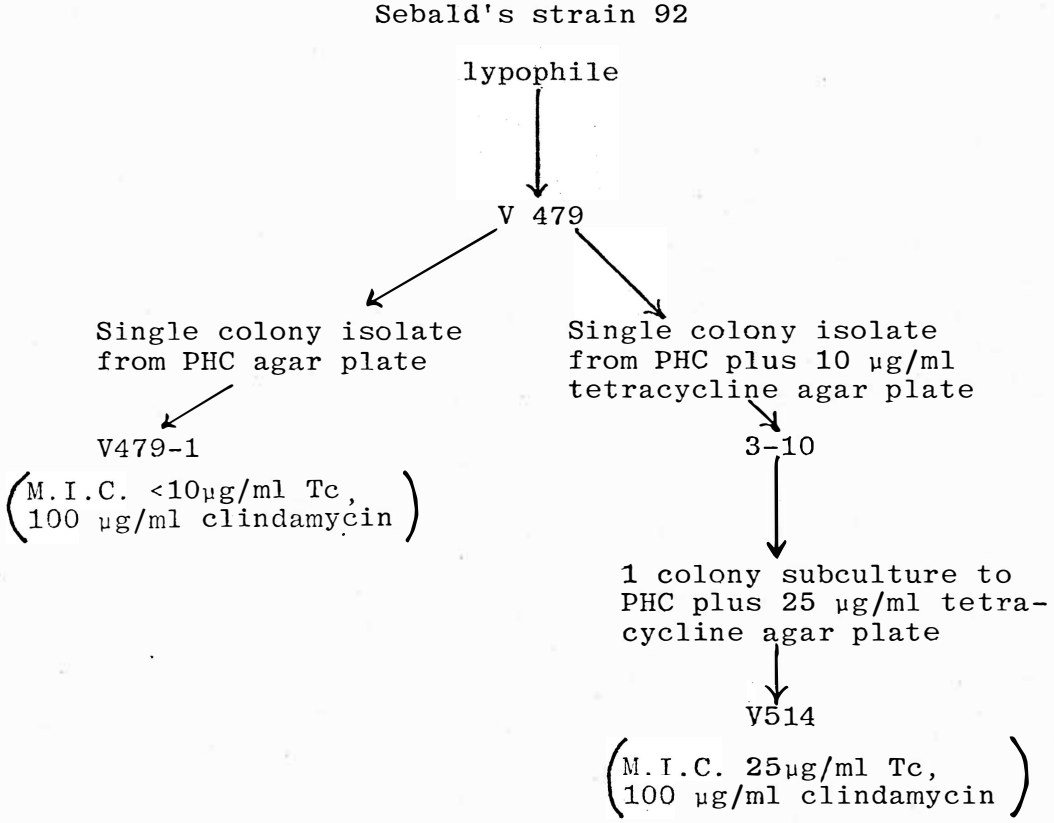
Bacteroides fragilis V479 (Sebald's 92) was tested for obligate anaerobic growth, gram stain reaction, indole production, catalase production and acid production from various carbohydrates using the protocols described in the VPI Anaerobe Manual (39). V479 proved to be an obligately anaerobic, irregularly shaped gram negative rod which was negative for indole production, positive for catalase production and produced an acid pH (<5.8) when glucose, fructose, raffinose, sucrose and xylose were incorporated into a peptone-yeast extract broth. No acid was produced when arabinose, inositol and mannitol were used. These results were consistant with the classification of *Bacteroides fragilis*.

Antibiotic sensitivity testing yielded the following minimal inhibitory concentrations (M.I.C.) for V479; 500 µg/ml erythromycin, 100 µg/ml clindamycin and <10µg/ml tetracycline. Sebald had reported that strain 92 was tetracycline resistant thus prompting the subculture of several of the colonies appearing on the 10 µg/ml tetracycline PHC agar plates used in the M.I.C. testing. These colonies were streaked to PHC agar plates containing 25 µg/ml tetracycline. The clone designated 3-10 survived the subculture process and yielded colonies of typical size and appearance. This

clone was randomly chosen among several that survived the subculture in higher tetracycline concentration and was given the final laboratory designation V514. M.I.C. testing was repeated using a single colony isolate of 479 designated V479-1 and the tetracycline resistant clone of V479, V514. Both V479-1 and V514 retained the same M.I.C. for erythromycin and clindamycin that had been seen for V479. V479-1 still demonstrated the tetracycline M.I.C. of less than 10 µg/ml where V514 showed an M.I.C. of 25 µg/ml tetracycline. Figure 1 is a schematic representation of the derivation of V479-1 and V514.

Figure 1

STRAIN PEDIGREE



Transfer Studies

Strains V479-1 and V514 were tested for their ability to transfer their respective antibiotic resistances to a spontaneously occurring rifampicin resistant derivative of B. uniformis V218 (VPI no. 0061-1). This rifampicin resistant recipient, designated V528, was mixed separately with each of the prospective donor strains using the filter mating protocol described in the Materials and Methods. The data shown in Table 5 indicate interspecies transfer of resistance to erythromycin, clindamycin and lincomycin from B. fragilis V479-1 to B. uniformis V528 (Cross 1). It is also shown that V479-1, as expected from its antibiogram, cannot serve as a donor of tetracycline resistance. Colonies recovered in successful matings are shown to be competent donors of the antibiotic resistance to a second recipient, B. fragilis V531 (Cross 2). Controls were always included in which comparable numbers of donor or recipient cells alone were taken through the same mating procedure and plated onto selective media. These controls showed no growth except for occasional instances where several colonites of the donor V479-1 would appear. These colonies represented spontaneous rifampicin resistant variants of V479-1 and accounted for only 0.1% of the colonies observed on the media used for plating the mating mixtures. The recipient phenotype of the drug resistant progeny was verified by their characteristic responses in the catalase and indole tests and by rifampicin sensitivity in the case

Table 5

Transfer Frequencies for V479-1

	Donor	Recipient	Antibiotics used in selection	Transfer frequency (drug resistant progeny per input donor cell
Cross 1	*B. fragilis* V479-1	*B. uniformis* V528	Cc, Rf	$7.6(\pm 2.2) \times 10^{-7}$ (a)
			Ln, Rf	$1.1(\pm 0.23) \times 10^{-6}$ (b)
			Em, Rf	$1.2(\pm 0.16) \times 10^{-6}$ (b)
			Cc, Ln, Em, Rf	$7.6(\pm 4.1) \times 10^{-7}$ (a)
			Tc, Rf	0
Cross 2	*B. uniformis* V544 (c)	*B. fragilis* V531	Cc, Ln, Em, Ap	$4.9(\pm 0.7) \times 10^{-6}$ (d)

(a) = Average of four separate experiments
(b) = Average of three separate experiments
(c) = Cc, Ln, Em resistant progeny of Cross 1
(d) = Average of two separate experiments

of Cross 2. Each donor and recipient pair was tested reciprocally for the production of bacteriocin-like activity using the standard overlay method. No such activity was detected in any of the assays.

Table 6 presents the transfer data for V514 with the standard recipient V528. V514 can be seen to transfer lincosamide-macrolide resistance but in addition, it can act as a donor of tetracycline resistance. Drug resistant progeny in this cross were also examined for the appropriate responses in the catalase and indole tests.

Evidence implicating cell to cell contact in the transfer process

Experiments designed to elucidate whether transformation, transduction or conjugation was responsible for the lincosamide-macrolide resistance transfer were undertaken using V479-1 and the standard V528 recipient. Cell-free filtrates of donor cultures prepared using either nitrocellulose (Millipore Corp., HA type, pore size 0.45 μm) or polycarbonate (Nucleopore corp., Pleasanton, La., pore size 0.40 μm) filter membranes could not promote the transfer of the antibiotic resistance. The chloroform-treated supernatant fluid of pelleted donor cells was mixed with recipient cells and this mixture was incubated under nitrogen gas for one hour at 37^{o}C. The mixture then was collected by suction onto mating filters and then treated in the normal fashion. Under these conditions the transfer of lincosamide-macrolide

Table 6

Transfer Frequencies for V514

Donor	Recipient	Antibiotics used in selection	Transfer frequency (drug resistant progeny per input donor cell) [a]
V514 [b]	V528	Cc, Em, Rf	$1.2\ (\pm 2.1) \times 10^{-6}$
		Tc, Rf	$2.3\ (\pm 7.5) \times 10^{-5}$
		Cc, Em, Tc, Rf	$1.6\ (\pm 1.6) \times 10^{-6}$ [b]

(a) = Average of three separate experiments

(b) = Cc^r, Em^r, Tc^r, Rf^r progeny isolated, given V601 designation

resistances was not seen to occur. DNase (Sigma) was added to separate liquid cultures of the donor and recipient cells at a final concentration of 10 µg/ml and these mixtures incubated for one hour at 37°C. The donor and recipient cells then were collected and mixed using the normal mating protocol with the exception that 0.1 ml of 100 µg/ml DNase was spread onto the PHC agar mating surface. Under these conditions there was anywhere from a three-fold to a ten-fold decrease in the lincosamide-macrolide resistance transfer. However, a similar lowering of the transfer frequency was observed when the DNase buffer (0.005 M $MgSO_4$) alone was added to the mating mixture and media indicating that the transfer event was not sensitive to the action of DNase.

To judge the necessity of cell-to-cell contact, matings were performed in which donor and recipient cells were separated by membrane filters. The donor cells alone were collected on either nitrocellulose or polycarbonate filters. These filters then were placed cell-side-up directly onto filters on which the recipient has been collected. The filter "sandwiches" were incubated 24 hours on PHC agar. These cells were collected as described under Materials and Methods and plated onto selective media. In no case was the transfer of antibiotic resistance observed from cells treated in this fashion.

Transfer of resistance was not observed when donor and recipient strains were mixed in liquid PHC broth culture and anaerobically incubated for 24 hours.

Table 7 presents a summary of data from experiments designed to elucidate the nature of the genetic transfer event which is responsible for lincosamide-macrolide resistance transfer by V479-1.

Recipient survey

A limited survey of Bacteroides strains which would serve as recipients of lincosamide-macrolide resistance from V479-1 was undertaken. The results of the survey are presented in Tables 8 and 9. Spontaneous rifampicin resistant variants of the strains with VPI designations listed in the second column of Tables 8 and 9 were isolated and given the V number designations listed in the first column of the tables. These rifampicin resistant prospective recipients then were mated with V479-1 and clindamycin resistant progeny scored as described in the Materials and Methods. Each mating was performed at least twice with the exception of V535 which was attempted only once. Table 8 lists the 7 strains which proved to act as recipients of clindamycin resistance and Table 9 lists the 12 strains for which transfer of clindamycin resistance was undetectable. It should be noted that the V479-1 x V528 mating pair was always included in these transfer studies as a positive control for transfer.

Table 7

Summary of evidence implicating the necessity for cell-to-cell contact in lincosamide-macrolide resistance transfer by *B. fragilis* strain V479-1

Transfer conditions	Frequency of *B. uniformis* lincosamide-macrolide resistant progency per input donor cell[a]
A) Standard transfer (V479-1 x V528)	1.1×10^{-6}
B) DNase in mating mixture and spread on agar mating surface	2.2×10^{-7}
C) DNase buffer alone as in B	3.4×10^{-7}
D) Filtrate of V479-1 mixed with recipient	None detectable[b]
E) Chloroform-treated supernatant of V479-1	None detectable
F) Donor and recipient cells separated by membrane filter	None detectable
G) Donor and recipient cells mixed and incubated in liquid culture 24 hours	None detectable

(a) These frequencies are from a single experiment but representative of data acquired in two separate experiments.

(b) Level of detection = 10^{-9}

Table 8

Lincosamide - Macrolide Resistance Transfer by *Bacteroides Fragilis* V479-1: Host Range Study

Laboratory Designation	VPI Designation	*Bacteroides* Species	G+C Mole Fraction of Recipient Strain (46)	Range of Frequencies of LM^R Transfer (LM^R Progeny/input Donor)
V529	2553	*fragilis* - 1	42	10^{-6}
V528	0061-1	*uniformis*	46	10^{-5} - 10^{-7}
V700	C54-2	*fagilis* subsp. A	45	10^{-6}
V701	2302	*thetaiotaomicron*	42	10^{-5} - 10^{-6}
V702	2822	*ovatus*	40	10^{-7}
V567	0038-1	*ovatus*	40	10^{-6}
V532	B-70	*fragilis*	42-44	10^{-6}

Table 9

Lincosamide-Macrolide Resistance Transfer by *Bacteroides Fragilis* V479-1: Host Range Study

Strains Where LM^R Transfer is not Detectable ($<10^{-8}$ LM^R/Input Donor)

Laboratory Designation	VPI Designation	*Bacteroides* Species	G+C Mole Fraction of Recipient Strain (46)
V530	4245	*vulgatus*	41
V535	4533 C	*oralis*	47
V703	C51-27	*uniformis*	46
V704	C20-25	*uniformis*	45-47
V705	JA-2	*uniformis*	45-57
V706	J7-54	"3452-A"	40-42
V707	2308	"3452-A"	41
V708	8608	"3452-A"	40
V709	C50-2	*distasonis*	44
V710	C30-45	*distasonis*	44
V711	C41-35	*ovatus*	40
V712	6947	*fragilis* - 1	42

Kinetics of resistance transfer

To assess the time course of lincosamide-macrolide resistance transfer, a rifampicin resistant mutant of B. fragilis V531 (VPI no. B-70) was isolated. This strain designated V532 was used as a recipient in transfer experiments with B. fragilis V545 (lincosamide-macrolide resistant progeny from Cross 2 seen in Table 5), thus yielding an isogenic mating system (V531 genetic background).

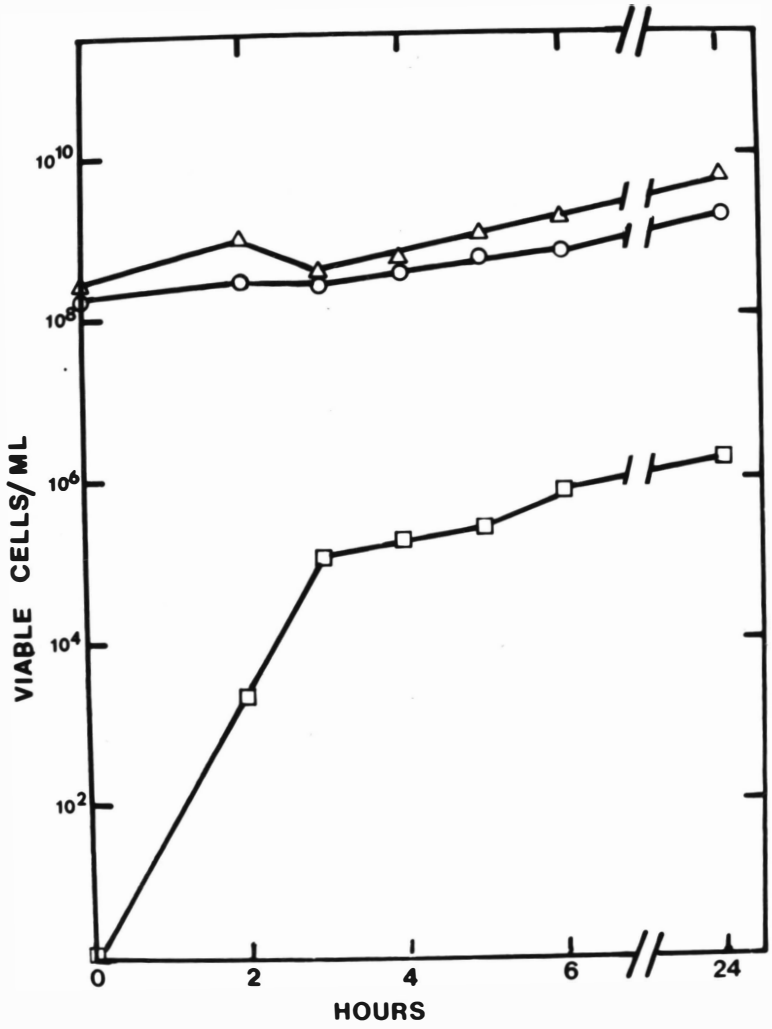
As can be seen in Figure 2, transcipients are detected (1.1×10^{-5} drug resistant progeny/input donor cell) at 2 hours following mixing of donor and recipient cells. This level of drug resistant progeny increases to about 3.3×10^{-4} drug resistant progeny/input donor cell at 3 hours; thereafter, increase in numbers of drug resistant progeny parallels the growth of donors and recipients in the mating mixture. Three separate experiments of this type, using V545 and V532 gave similar results. As seen in Figure 2, the frequency of drug resistant progeny/input donor cell is approximately 7.5×10^{-3} at 24 hours representing a 1000-fold elevation over the transfer frequencies seen in interspecies (non-isogenic) matings (see Tables 5 and 8).

Figure Legend

Figure 2. Kinetics of resistance transfer. Seven individual mating mixtures (V545 was the donor and V532 the recipient) were collected onto nitrocellulose filters simultaneously and incubated separately in Gas-Pak anaerobic jars. At each time point, the respective jar was opened and the cells were washed from the filters as described under Materials and Methods. These cell suspensions then were appropriately diluted and plated onto media enabling the enumeration of donors(○), recipients (△), and progeny (□).

Figure 2

Kinetics of Resistance Transfer



Demonstration of plasmid-mediated antibiotic resistance transfer

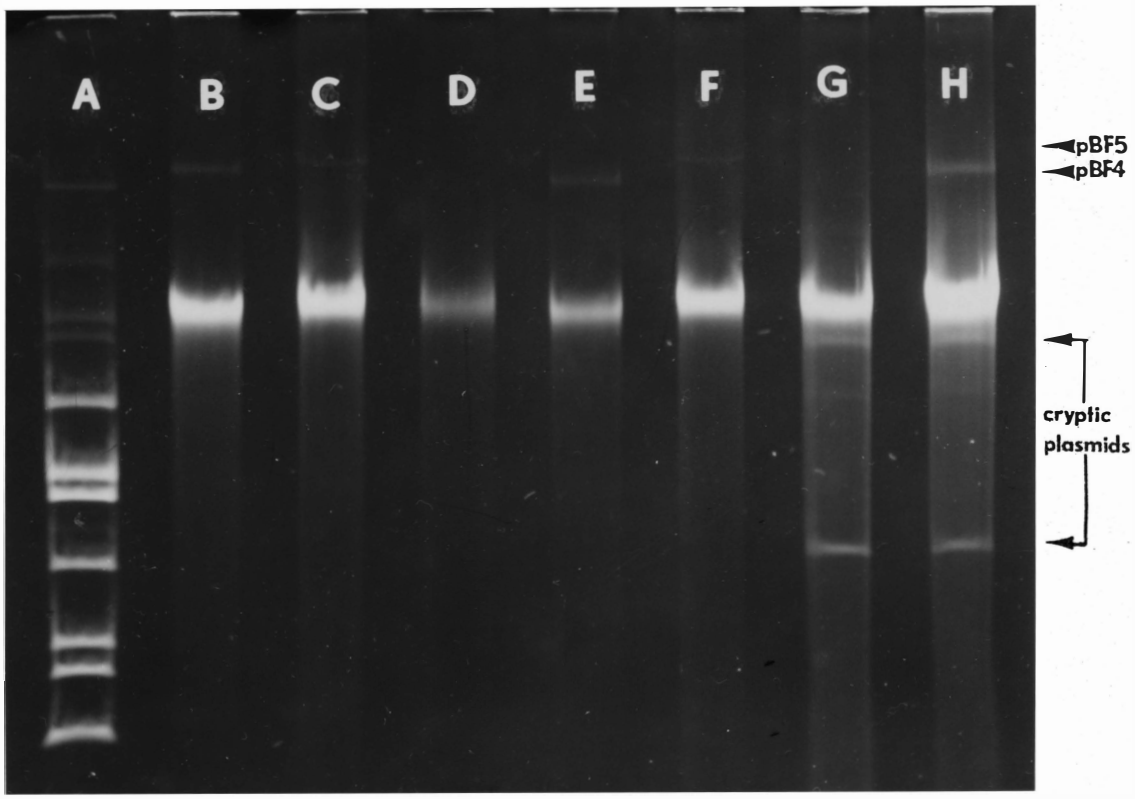
The observation that strains V479-1 and V514 could serve as donors of antibiotic resistance lead to the examination of those strains and their respective drug resistant progeny for plasmid DNA. The mini-lysate technique (see Materials and Methods) was utilized in isolating plasmid DNA from these strains. The results of this examination can be seen in Figure 3. Both strains V479-1 and V514 can be seen to possess single plasmid species. The plasmid of V479-1 designated pBF4 can be seen to migrate further than the plasmid,pBF5 of strain V514. The respective drug resistant progeny of V479-1 and V514 (lanes E and F) can be seen to contain plasmid DNA apparently equal in size based on similar migration to their counterparts in lanes B and C. The secondary recipient, V531 can be seen to possess two small cryptic plasmids (lane G) and upon the transfer of lincosamide-macrolide resistance from V544, this plasmid background acquires a plasmid apparently equal in size to the pBF4 plasmid in V479-1 (lane B) and the plasmid of V544 (lane E).

Figure Legend

Figure 3. Agarose gel electrophoresis of Bacteroides plasmid DNA. Plasmid DNA isolated by the mini-lysate technique was applied to the top slots and electrophoresis was from top to bottom. Agarose concentration for this particular slab gel was 0.75%. Lane A is the CsCl-ethidium bromide gradient purified plasmid DNA from V517 which provides plasmid size references ranging from 1.4 to 36 Mdal. Lane B contains the plasmid DNA from V479-1; Lane C the plasmid DNA from V514; Lane D contains just the chromosomal DNA of the standard recipient strain V528 which as been shown to be plasmidless (101). Lane E contains the plasmid DNA from the lincosamide-macrolide resistant progeny V544, isolated from the 479-1 x V528 mating; Lane F plasmid DNA from V601, the lincosamide-macrolide and tetracycline resistant progeny from the V514 x V528 mating; Lane G the plasmid DNA from V531 which was the secondary recipient used in matings with V544; Lane H is the lincosamide-macrolide resistant progeny V545 from the V544 x 531 mating. The diffuse band in the center of lanes B throught H represents contaminating chromosomal DNA co-isolated with plasmid DNA in the mini-lysate technique.

Figure 3

Agarose gel electrophoresis of Bacteroides plasmid DNA



Physical characterization of pBF4 and pBF5

a) Size Analysis

Over the course of these studies numerous agarose electrophoretic gel determinations of plasmid size were performed with both CsCl-ethidium bromide gradient purified pBF4 and pBF5 DNA and preparations acquired by the mini-lysate technique. In addition, size analysis was performed by measuring the contour lengths of the open circular forms of the two plasmid species in the electron microscope. Lastly size analysis of the two plasmid species was also estimated by the addition of linear fragment sizes of plasmid DNA digested with the restriction endonuclease EcoRI. The size of these linear fragments was determined by their relative migration in agarose electrophoretic gels versus a known linear DNA standard.

A summary of the findings on the molecular size analysis using the three different experimental approaches can be seen in Table 10. Throughout the remainder of the thesis the sizes of pBF4 and pBF5 will be taken to be 27.2 and 59.8 Mdal respectively. These are the sizes as they were determined by contour length measurements.

Two representative electron micrographs of the open circular forms of pBF4 and pBF5 can be seen in Figures 4 and 5.

Table 10

Summary of plasmid size analysis

(Molecular size listed in Mdal)

	Electrophoretic Migration of CCC form	Contour Length	Summation of EcoRI fragments
pBF4	27.7 ± 1.9 (a)	27.2 ± 1.1 (c)	27.2 ± 1.2 (a)
pBF5	58.9 ± 3.6 (b)	59.8 ± 2.5 (c)	60.2 ± 2.1 (d)

(a) Based on 13 separate determinations
(b) Based on 10 separate determinations
(c) Based on measurement of ten separate molecules
(d) Based on 8 separate determinations

Figure legend

Figures 4 and 5.

Plasmid DNA purified by CsCl-ethidium bromide ultracentrifugation was nicked by gamma irradiation to produce open circular forms which were spread by the Kleinschmidt technique (52). The DNA was picked up on parlodium coated copper grids, stained with uranyl acetate and shadowed with pallidium: platinum (80:20). The grids then were photographed at 9,000 to 12,000 Xl magnification with a HS-7S Hitachi electron microscope. Figure 4 is a representative electronmicrograph of pBF4 from V479-1. Figure 5 is a photograph of pBF5 from V514. pSC185 served as an internal reference molecule in both preparations.

Figure 4

Electron micrograph pBF4

w/pSC185

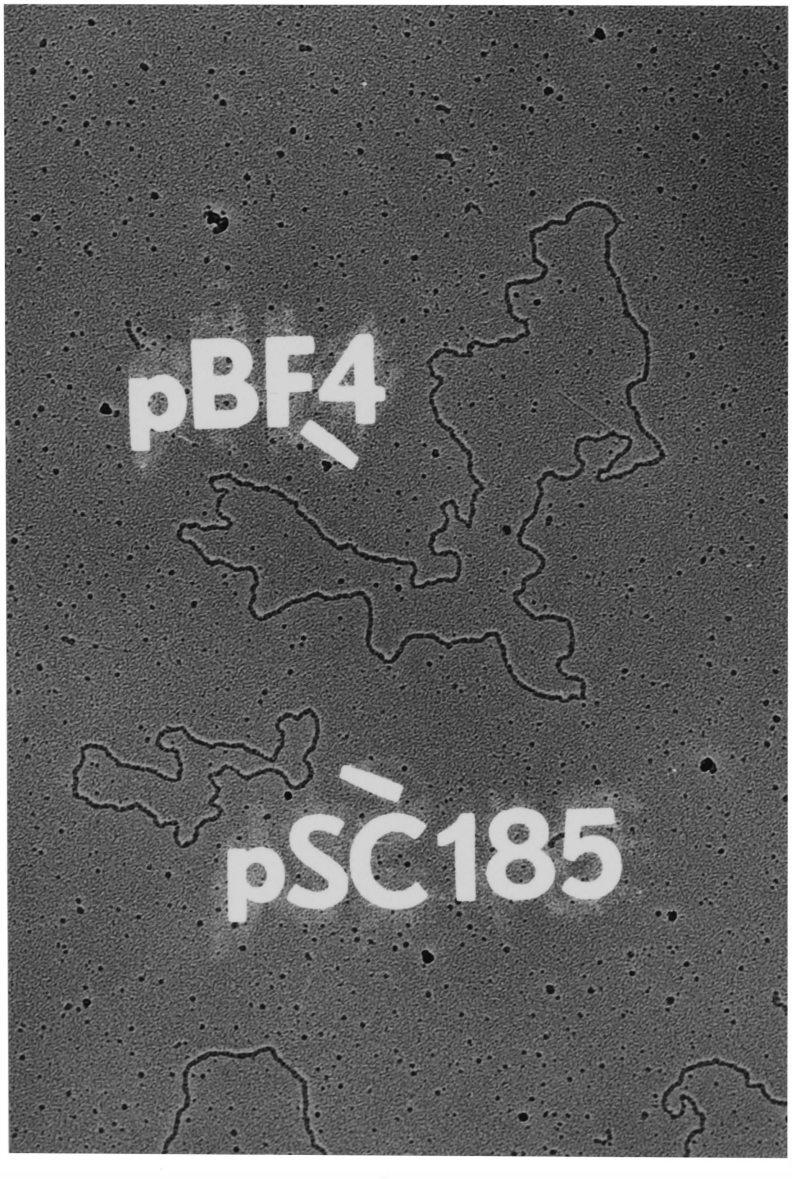


Figure 5

Electron micrograph

pBF5 w/pSC 185

b) Plasmid DNA quantitation

Bacteroides DNA radioactively labeled in vivo with ^{3}H-thymidine was isolated and subjected to CsCl-ethidium bromide ultracentrifugation. Fractions from the bottom of gradient were collected and the radioactivity measured by scintillation counting. The fractions representing the plasmid DNA were pooled as were the fractions representing the linear chromosomal fragments. ^{3}H-thymidine labeling of plasmid and chromosomal DNA in Bacteroides has been shown to be uniform (101). The Bacteroides chromosome has been estimated to be 2.5×10^{9} daltons in size (101). The percentage of the total radioactivity occurring in the pooled fractions representing plasmid DNA (CCC DNA) was multiplied by the molecular weight estimate for the Bacteroides genome. This resulted in an estimate of the amount of DNA isolated in the CCC form in these gradients. This estimate then was divided by the molecular sizes of two plasmids pBF4 and pBF5 to give an estimate of the number of copies of the respective plasmids per 2.5×10^{9} dalton chromosome equivalents. The results of this analysis can be seen in Table 11. These results represent a minimum copy number estimation since the open circular and linear forms of the pBF4 and pBF5 plasmids would band in the chromosomal portion of the gradient. The percentage of radioactivity they would contribute to the analysis is therefore lost from the plasmid equivalent and added to the chromosomal equivalent.

Hence, it appears that both pBF4 and pBF5 are in the low copy number category of bacterial plasmids.

Table 11

Plasmid DNA copy number

Plasmid	Size (Mdal)	% of Chromosome [a]	Copies/2.5 x 10^9 dal equivalents
pBF4	27	1.6 ± 0.3	1.5 ± 0.3
pBF5	60	1.9 ± 0.4	0.8 ± 0.2

(a) Based on three separate determinations

Molecular organization of Bacteroides plasmids

a) pBF4 and pBF5 sequence relationship.

Since pBF4 and pBF5 presumably arose from a single clinical isolate and occurred in strains phenotypically alike except for tetracycline resistance it was of interest to investigate the plasmid sequence relationship. Various restriction endonucleases were chosen to digest the two plasmids in an attempt to discern if fragments of similar size could be seen to occur in each plasmid. If particular restriction endonuclease cleavage sites were similarly spaced in each plasmid this would suggest that the two plasmids shared a common ancestry.

Figure 6 is a representative picture of an agarose electrophoretic gel of pBF4 and pBF5 digested with the restriction endonuclease EcoRI. Four of the EcoRI fragments of pBF4 are seen to have similarly sized counterparts in the pBF5 fragment pattern. Fragments B, C, E and F of pBF4 are seen to occur in pBF5. Fragment A of pBF4 and the largest fragment of pBF5 appear to be the same size and further data in this regard will be presented below.

Similar results for other restriction endonucleases such as Hind III, Xba I, Hpa I, Kpn I, BamHI and Ava I were seen. Although gel picutres of these digests will not be shown, it is apparent that by the criterion of restriction endonuclease fragment patterns, pBF5 is in fact made up of pBF4 plus an insertion of nearly 32 Mdal of additional DNA.

A summary of the number and size of the DNA fragments resulting from the different restriction enconucleases is presented in Tables 15 and 18 and will be discussed below.

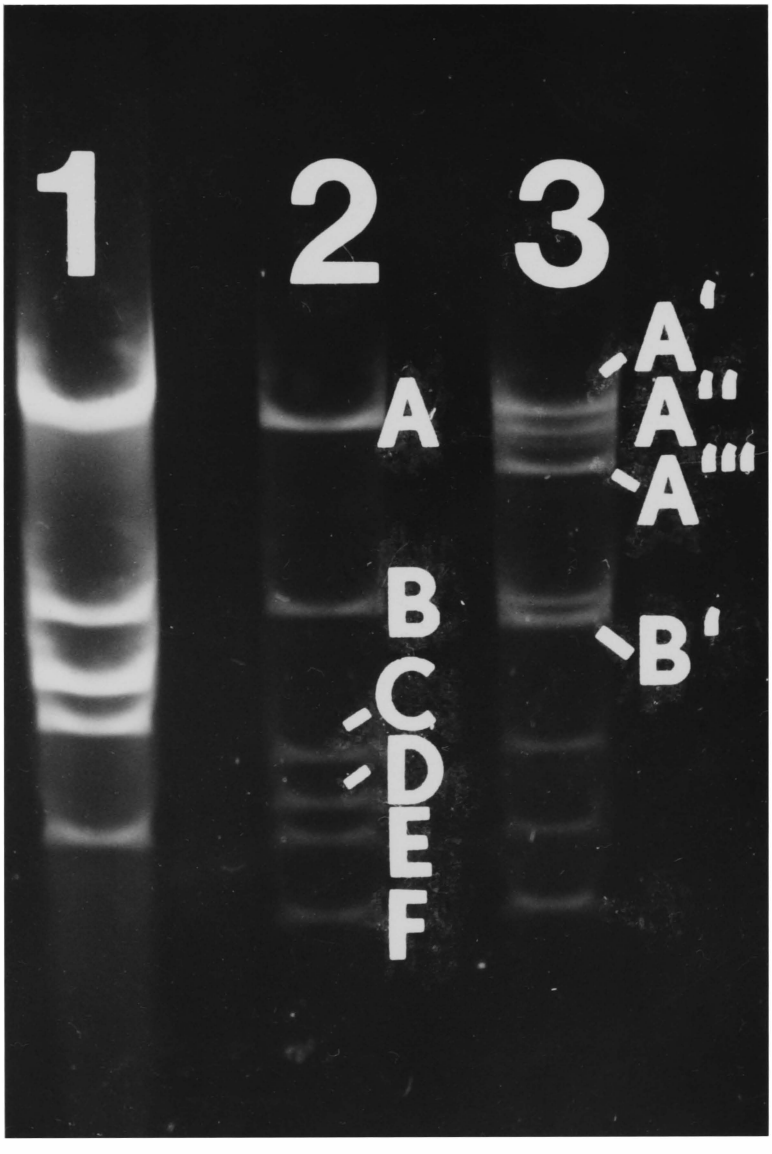
Figure legend

Figure 6. Agarose gel electrophoresis of pBF4 and pBF5 DNA digested with the restriction endonuclease EcoRI.

Plasmid DNA purified by CsCl-ethidium bromide ultracentrifugation was subjected to digestion with the restriction endonuclease EcoRI as described in the Materials and Methods. Lambda DNA acquired commercially was also digested with EcoRI. The EcoRI digested lambda can be seen in lane 1. EcoRI digested pBF4 and pBF5 can be seen in lanes 2 and 3 respectively. Agarose gel concentration for this particular slab was 0.8%.

Figure 6

EcoRI digest



b) Isolation and characterization of plasmid deletions

i) Isolation of lincosamide-macrolide sensitive variants of strains harboring either pBF4 or pBF5.

Experiments were undertaken in attempt to isolate lincosamide-macrolide sensitive variants of Bacteroides strains harboring the pBF4 and pBF5 resistance plasmids. Strains V479-1, V544 and V545 harboring pBF4 and V514 and V601 which harbor pBF5 were subjected to the screening procedure described in Materials and Methods for the isolation of drug sensitive variants. These experiments were carried out with the intent of screening any sensitive variants for the loss of plasmid DNA. Such experiments would provide an additional arguement supporting the notion that pBF4 and pBF5 are responsible for the lincosamide-macrolide resistance phenotype if drug sensitive variants can be shown to be "cured" of their respective plasmid DNA.

A number of clindamycin sensitive variants of the plasmid-harboring strains were found. All of these clindamycin sensitive variants also were found to have lost simultaneously the erythromycin resistance phenotype. A summary of these results using the pBF4 bearing strains can be seen in Table 12. Table 13 is a similar summary for the strains harboring pBF5. Strains V536, V537, V598, V599, V600 and V689 were checked for their spontaneous reversion to lincosamide-macrolide resistance. The procedure for testing this reversion is described in the Materials and Methods.

Table 12

Screening for loss of the lincosamide-macrolide (LM) resistance phenotype in strains harboring pBF4

Strain	Growth conditions prior to screening	Number of colonies per independent experiment tested	Number of LM sensitive colonies detected	LM sensitive strains isolated
V479-1	Growth at 37°C	232	0	-
		50	1 (a)	V599
	Growth at 42°C	530	0	-
		105	0	-
	Growth in presence of 0.005mM ethidium bromide	550	3 (a)	V598, V600 (b)
		250	0	-
		55	2	V688, V689
		58	0	-
		54	0	-
V544	Growth at 37°C	198	0	-
V545	Growth at 37°C	177	0	-

(a) Chronologically, V599, V598 and V600 were isolated at the same time. These isolations occurred nearly a year after V479-1 had been kept in stock culture in the absence of clindamycin.

(b) Third LM sensitive clone examined for plasmid content but not given V designation.

Table 13

Screening for loss of lincosamide-macrolide (LM) resistance phenotype in strains harboring pBF5

Strain/plasmid	Growth conditions prior to screening	Number of colonies tested in separate experiemnts	Number of LM sensitive colonies	LM sensitive strains isolated
V514/pBF5 (a)	Growth in presence of 0.005 mM ethidium bromide	428	12	V536, V537 V538
V514/pBF5 (b)	Growth at 37°C	181	55	V690,V691,V692,V693
V601/pBF5 (a)	Growth at 37°C	24	4	V694,V695,V696,V697
V601/pBF5 (a)	Growth in presence of 0.005 mM ethidium bromide	745	15	V698, V699

(a) The stock cultures for these strains were maintained in the presence of 1 µg/ml tetracycline.

(b) The stock culture in this case has been sub-cultured 8-10 times in the absence of any tetracycline over a year and a half period.

No revertant colonies were seen to spontaneously arise. Strains V536, V537 and V600 were tested to see if they would revert to the clindamycin resistance phenotype when grown in the presence of acridine orange, ethyl methyl sulfonate, hydroxylamine, ethidium bromide and nitrosoguanidine. No drug resistant revertant colonies were observed under these conditions.

ii) Plasmid content of lincosamide-microlide sensitive variants.

Where feasible all of the lincosamide-macrolide sensitive variants isolated in an individual experiment were examined for plasmid content. The mini-lysate technique in combination with agarose gel electrophoresis was employed for these screenings. The plasmid content of each of the sensitive strains was compared to the plasmid content of its respective resistant parent.

All six of the lincosamide-macrolide sensitive derivatives of V479-1 were screened for plasmid DNA. Four of the six sensitive clones were free of plasmid DNA detectable by the mini-lysate-agarose gel procedure. The remaining two sensitive clones (V600 and V689) were isolated independently and seen to harbor plasmids smaller in size than the parental pBF4 plasmid.

No plasmid-free, lincosamide-macrolide sensitive derivatives of strains originally harboring pBF5 were observed. However, the frequency of lincosamide-macrolide sensitive

derivatives of these strains harboring plasmids smaller than pBF5 is much greater than in the case for pBF4.

Presented in Table 14 is a summary of the plasmid content occurring in lincosamide-macrolide sensitive variants of strains harboring pBF4 or pBF5. It should be noted that any pair of strains seen to have plasmids similar in size were independently isolated.

Presented in Figure 7 is a photograph of an agarose gel demonstrating the plasmid DNA or plasmid-free state of each of the lincosamide-macrolide sensitive clones listed in Table 14.

Hereafter, the smaller plasmids found in the lincosamide-macrolide sensitive strains will be referred to as deletion derivatives of pBF4 of pBF5. Evidence for the deletion event will follow in the text.

Table 14

Summary of plasmid content in LM sensitive variants of LM resistant strains V479-1, V514 and V601

Parent Strain	Plasmid (Size, Mdal)	LM Sensitive Variant	Plasmid (Size, Mdal)[a]	Isolation Procedure: Spontaneous	Isolation Procedure: Growth in Ethidium Bromide
V479-1	pBF4 (27.2)	V598	Plasmid-free		X
		V599	Plasmid-free	X	
		V600	pBF4ΔLM-1 (21.7)		X
		V689	pBF4ΔLM-2 (25.6)		X
V514	pBF5 (59.8)	V537	pBF5ΔLM-1 (53)		X
		V690	pBF5ΔLM-2 (30.5)	X	
		V691	pBF5ΔLM-3 (35.5)	X	
		V693	pBF5ΔLM-4 (28)	X	
V601	pBF5 (59.8)	V694	pBF5ΔLM-5 (19.4)	X	
		V695	pBF5ΔLM-6 (36.2)	X	
		V697	pBF5ΔLM-7 (22.8)	X	
		V698	pBF5ΔLM-8 (36.0)		X
		V699	pBF5ΔLM-9 (20.6)		X

(a) Size estimates made by comparing the migration distances of these plasmids to the migration distance of plasmids of known molecular size in agarose gel electrophoresis as detailed in the Materials and Methods.

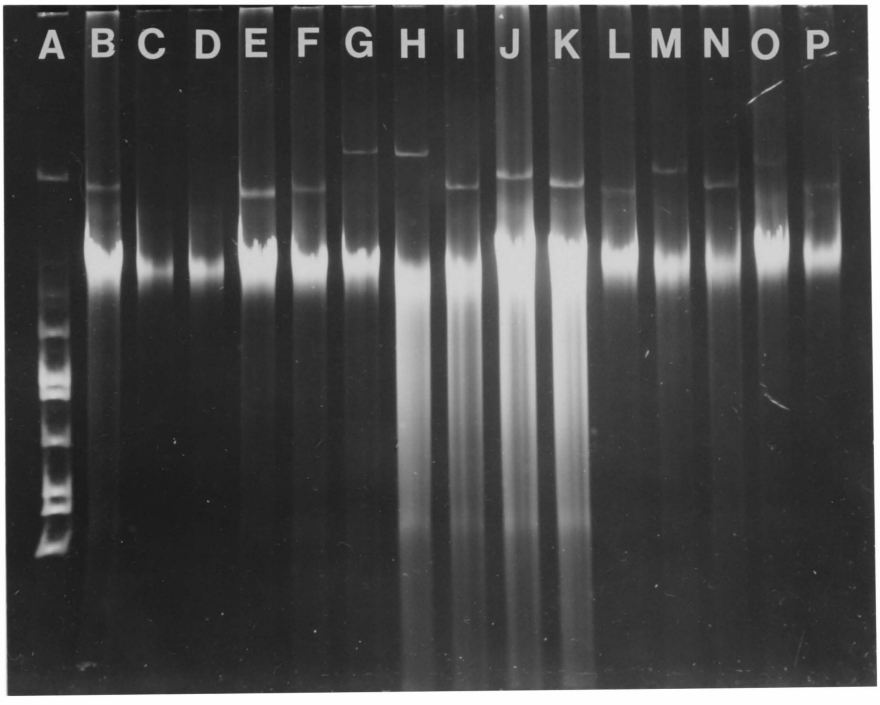
Figure legend

Figure 7. Agarose slab gel electrophoresis of plasmid DNA from lincosamide-macrolide sensitive derivatives of strains V479-1, V514 and V601.

Each lincosamide-macrolide sensitive derivative as well as the parental strains V 479-1 and V514 were subjected to the mini-lysate technique for isolation of plasmid DNA. Agarose concentration was 0.7%. Lane A contains the standard reference plasmid molecules of E. coli strain V517 which vary in size from 1.4 to 35.8 Mdal. The order of strains is as follows: lane B, V479-1 (pBF4); lane C, V598; lane D, V599; lane E, V600; lane F, V689; lane G, V514 (pBF5); lane H, V537; lane I, V690; lane J, V691; lane K, V693; lane L, V694; lane M, V695; lane N, V697; lane O, V698 and lane P, V699.

Figure 7

Agarose slab gel electrophoresis of plasmid DNA



iii) Restriction endonuclease digest analysis of plasmid DNA from LM sensitive derivatives of V479-1 and V514.

With the isolation of smaller derivatives of pBF4 and pBF5 it was of interest to see what sequence rearrangements had occurred with these plasmids. Restriction endonuclease fragment analysis was carried out with the plasmids from strains V600 (pBF4ΔLM-1), V689 (pBF4ΔLM-2) and V537 (pBF5 ΔLM-1). These particular strains were chosen because they represented the cases where the smallest change in plasmid size had occurred. As apparent deletion derivatives of pBF4 and pBF5, these would provide the best opportunity for localizing the lincosamide-macrolide resistance gene to a specific restriction endonuclease fragment. Plasmid DNA from these strains was purified by CsCl-ethidium bromide centrifugation, digested with different restriction endonucleases and the digests subjected to slab gel electrophoresis along with digests of the parental plasmids. In all three cases the digest fragment patterns confirmed that the smaller plasmids were derivatives of either pBF4 or pBF5. A majority of the digest fragments from the smaller plasmids were equal in size to the digest fragments of the respective parental plasmids. Additionally, it was observed that common digest fragments from the parental plasmids were missing in the digests of the three deletion plasmids. Figure 8 is a photograph of a representative EcoRI digest of the plasmids pBF4, pBF4ΔLM-1 and pBF4ΔLM-2. pBF4ΔLM-1 has fragments B,

C and E in common with pBF4. pBF4ΔLM-2 has fragments A, B, C, E and F in common with pBF4. The molecular size of the EcoRI fragments of pBF4 and the two deletion derivatives will be presented later in the results section.

Figure 9 is a picture of an agarose electrophoretic gel showing the representative Hind III digest of the pBF4, pBF4ΔLM-1, pBF5 and pBF5ΔLM-1 plasmids. In both cases, the A fragment of pBF4 and pBF5 is absent in the Hind III digests of the plasmids contained in the lincosamide-macrolide sensitive derivatives of V479-1 and V-514. The new B' fragment seen in lane C represents the case where the deletion has shortened the distance between Hind III cleavage site resulting in a new smaller Hind III fragment in pBF4ΔLM-1.

Figure legend

Figure 8. Agarose gel electrophoresis of pBF4, pBF4ΔLM-1 and pBF4ΔLM-2 DNA digested by EcoRI.

Agarose concentration was 0.7%. Lane D contains the EcoRI digest fragments of lambda DNA used as a linear DNA reference standard. Lane A is pBF4 digested with EcoRI. Lane B contains pBF4ΔLM-1 digested with EcoRI and Lane C has the pBF4ΔLM-2 EcoRI digest. Fragments are ordered A, B, C, D, E and F going from the top to the bottom in lane A.

Figure 8

EcoRI digest

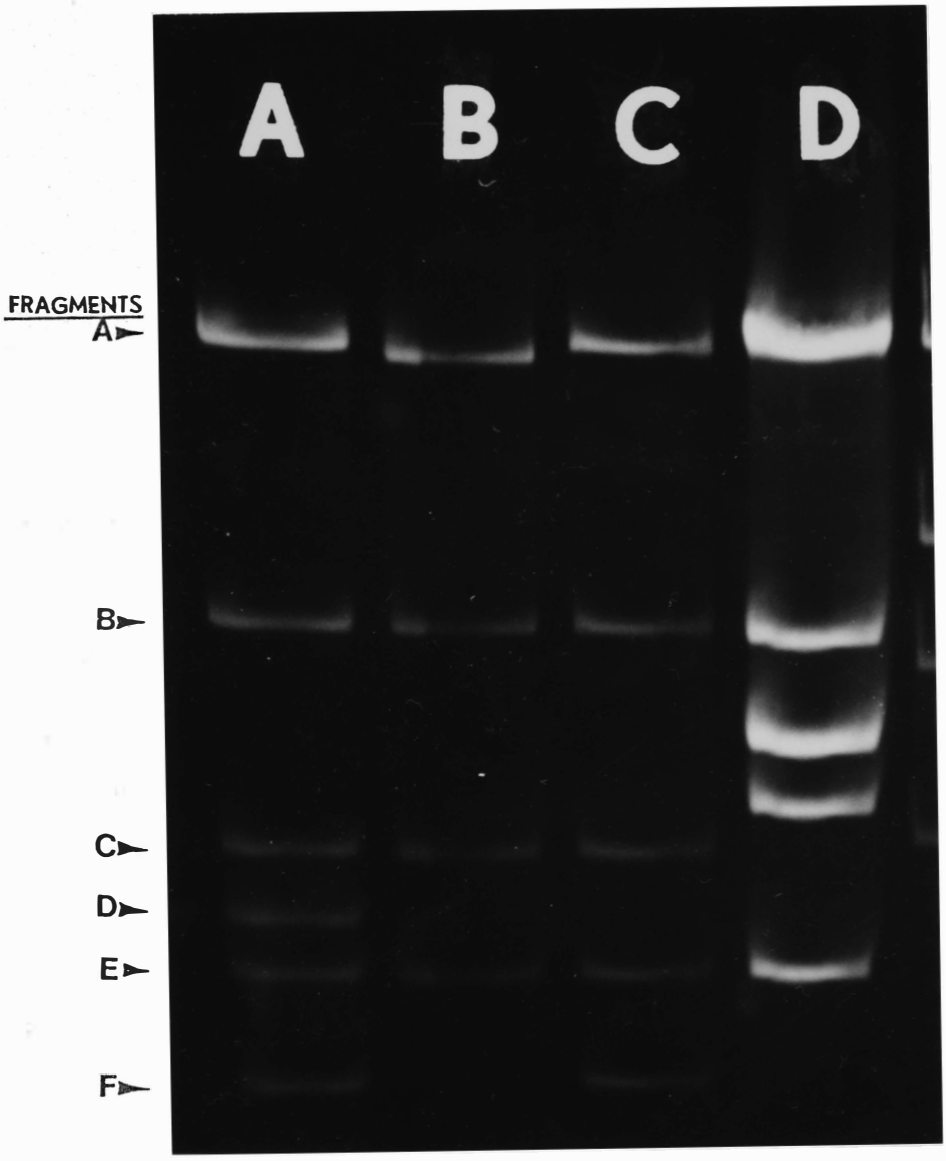


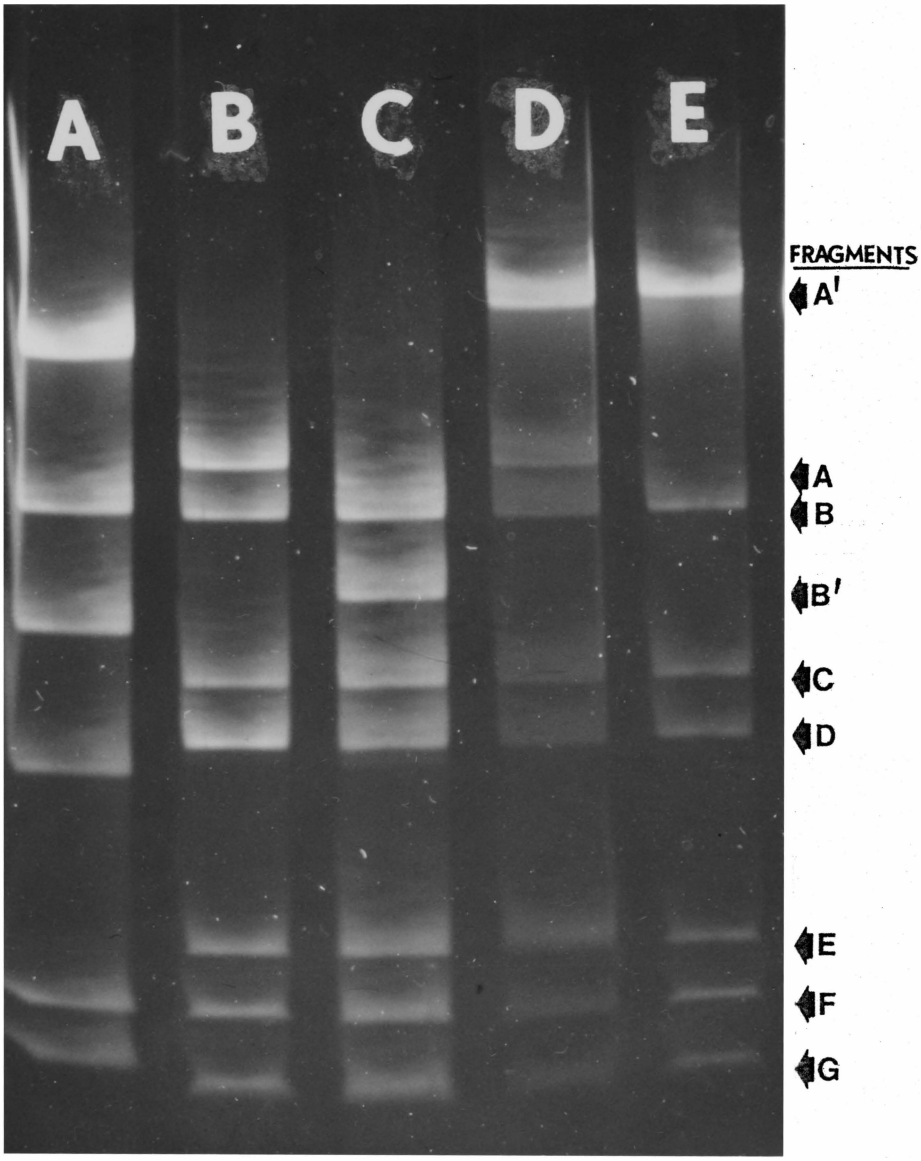
Figure legend

Figure 9. Agarose gel electrophoresis of pBF4, pBF4ΔLM-1, pBF5 and pBF5ΔLM-1 DNA digested with Hind III.

Agarose concentration was 0.7%. Lane A is lambda digested with Hind III which serves as the reference standard of linear DNA. Lanes B, C, D and E respectively contain pBF4, pBF4ΔLM-1, pBF5 and pBF5ΔLM-1 DNA digested with Hind III. Densitometry revealed that fragment D of pBF4, A' of pBF5 and pBF5ΔLM-1, and F of pBF5ΔLM-1 were doublet species. Fragments are ordered A', A, B, C, D, E, F, and G going from top to bottom in lane D.

Figure 9

Hind III digest



Restriction endonuclease fragment size analysis and fragment mapping of pBF4.

Tables 15, 16, 17 and 18 present the respective summaries of restriction endonuclease fragment sizes for pBF4, pBF4ΔLM-1, pBF4ΔLM-2 and pBF5. Fragments generated by a single enzyme from each of the plasmids were given common letter designations if their migration distances were indistinguishable. The (') designation given to fragments of the pBF4 deletion plasmids and the pBF5 plasmid indicates that the particular fragment migrates different but closest to a pBF4 fragment of the same letter designation.

Using the data on fragment sizes, a physical map of pBF4 was generated with the fragments of Hpa I digestion. This was made possible by piecing together the complete digest fragments with the partial digest fragments seen in Hpa I digests of the pBF4ΔLM-2 plasmid. For pBF4ΔLM-2 fragments B' and C added together give a total of 8.6 Mdal. Fragments C and D give a total of 7.5 Mdal. The two partial digest fragments seen in incomplete digests of pBF4ΔLM-2 are 8.3 ± 0.3 and 6.9 ± 0.6 Mdal respectively. The deletion event observed for pBF4ΔLM-1 and the Hpa I digest fragments from this plasmid indicate that Hpa I fragments A and B must be contiguous in pBF4. Therefore, the partial digest fragments seen in pBF4ΔLM-2 Hpa I digests must represent the case where fragment C is contiguous to fragment B' and fragment D is contiguous to Fragment C. The possibility that partial digest fragment B represents

Table 15

Summary of restriction endonuclease fragment sizes for pBF4

Restriction endonuclease	Cleavage Site (BRL Catalog)	Fragment	Fragment Size (Mdal)	Summation of Fragment Sizes
EcoRI	5'↑GAATTC 3'	A	13.0 ± 0.7	27.2 ± 1.2 (a)
		B	5.2 ± 0.3	
		C	2.8 ± 0.1	
		D	2.4 ± 0.1	
		E	2.1 ± 0.1	
		F	1.7 ± 0.2	
Hind III	5'↑AAGCTT 3'	A	7.8 ± 0.3	28.7 ± 1.8 (b)
		B	6.4 ± 0.3	
		C	3.7 ± 0.4	
		D	3.1 ± 0.3 (doublet)	
		E	1.7 ± 0.1	
		F	1.5 ± 0.1	
		G	1.2 ± 0.1	
Hpa I	5'GTT↑AAC 3'	A	13.3 ± 0.3	27.9 ± 0.9 (c)
		B	7.0 ± 0.4	
		C	4.8 ± 0.3	
		D	2.9 ± 0.1	
Xba I	5'↑TCTAGA 3'	A	10.9 ± 1.0	30.3 ± 2.7 (d)
		B	10.3 ± 1.0	
		C	4.9 ± 0.4	
		D	4.2 ± 0.4	
Ava I	5'C↑PyCGPuG 3'	A	24.8	27.2 ± 1.1 (e)
		B	2.4 ± 0.1	
Kpn I	5'GGTAC↑C 3'	A	27.2 ± 1.1	27.2 ± 1.1 (e)
Bam HI	5'G↑GATCC 3'	A	27.2 ± 1.1	27.2 ± 1.1 (e)
Sal I	5'G↑TCGAC 3'	-	No cleavage site	
Sma I	5'CCC↑GGG 3'	-	No cleavage site	

(a) Based on 13 separate digests
(b) Based on 7 separate digests
(c) Based on 4 separate digests
(d) Based on 3 separate digests
(e) Based on 2 digests, largest linear fragment size estimated by size of DNA needed to total contour length of pBF4.

Table 16

Summary of restriction endonuclease fragment sizes for pBF4ΔLM1

Restriction endonuclease	Cleavage Site (BRL Catalog)	Fragment	Fragment Size (Mdal)	Summation of Fragment Sizes
EcoRI	5'G↑AATTC 3'	A''''	12.5 ± 1.2	22.6 ± 1.5 (a)
		B	5.1 ± 0.4	
		C	2.8 ± 0.1	
		E	2.1 ± 0.1	
		Partial digest fragment a	8.6 ± 0.3 (b)	
		Partial digest fragment b	6.9 ± 0.2 (c)	
Hpa I	5'GTT↑AAC3'	A'	13.8 ± 0.4	21.3 ± 0.1 (d)
		C	4.7 ± 0.3	
		D	2.8 ± 0.1	
		Partial digest fragment b	6.9 ± 0.6 (d)	
Ava I	5'C↑PyCGPuG3'	A'	21.7 (e)	
Kpn I	5'GGTAC↑C3'	A'	21.7 (e)	
BamHI	5-G↑GATCC3'	A'	21.7 (e)	

(a) Based on 7 separate digests
(b) Based on 8 separate digests
(c) Based on 6 separate digests
(d) Based on 3 separate digests
(e) Single fragment evident in digests, molecular size taken to be the size as judged by contour length measurements.

Table 17

Summary of restriction endonuclease fragments of pBF4ΔLM-2

Restriction endonuclease	Cleavage Site	Fragment	Fragment Size (Mdal)	Summation of Fragment Sizes
EcoRI	5'G↑AATTC3'	A	13.1 ± 0.6	24.8 ± 0.9 (a)
		B	5.3 ± 0.3	
		C	2.8 ± 0.1	
		E	2.1 ± 0.1	
		F	1.6 ± 0.1	
Hpa I	5'GTT↑AAC3'	A	13.1 ± 0.2	24.5 ± 0.2 (b)
		B'	3.9 ± 0.1	
		C	4.7 ± 0.3	
		D	2.8 ± 0.1	
		Partial digest fragment a	8.3 ± 0.3	
		Partial digest fragment b	6.9 ± 0.6	
Ava I	5'C↑PyCGPuG3'	A''	Only single linear fragment detectable (c)	
Kpn I	5'GGTAC↑C3'	A''	Only single linear fragment detectable (c)	
Bam HI	5'G↑GATCC3'	A''	Only single linear fragment detectable (c)	

(a) Based on 7 separate digests
(b) Based on 3 separate digests
(c) Based on 2 separate digests

Table 18

Summary of restriction endonuclease fragment sizes for pBF5

Restriction endonuclease	Cleavage Site (BRL Catalog)	Fragment	Fragment Size (Mdal)	Summation of Fragment Sizes (Mdal)
EcoRI	5'G↑AATTC3'	A'	13.7 ± 0.8	60.2 ± 2.1 (a)
		A"	12.1 ± 0.8	
		A"'	9.9 ± 0.8	
		B	5.3 ± 0.2	
		B'	4.8 ± 0.2(doublet)(b)	
		C	2.9 ± 0.1	
		E	2.2 ± 0.2	
		F	1.7 ± 0.3(doublet)(b)	
		G	1.1 ± 0.3	
Hind III	5'A↑AGCTT3'	A'	16.4 ± 1.5(doublet)(b)	60.0 ± 3.4 (c)
		A	7.8 ± 0.3	
		B	6.4 ± 0.3	
		C	3.7 ± 0.4	
		D	3.1 ± 0.3	
		E	1.7 ± 0.1(doublet)(b)	
		F	1.5 ± 0.1	
		G	1.2 ± 0.1	

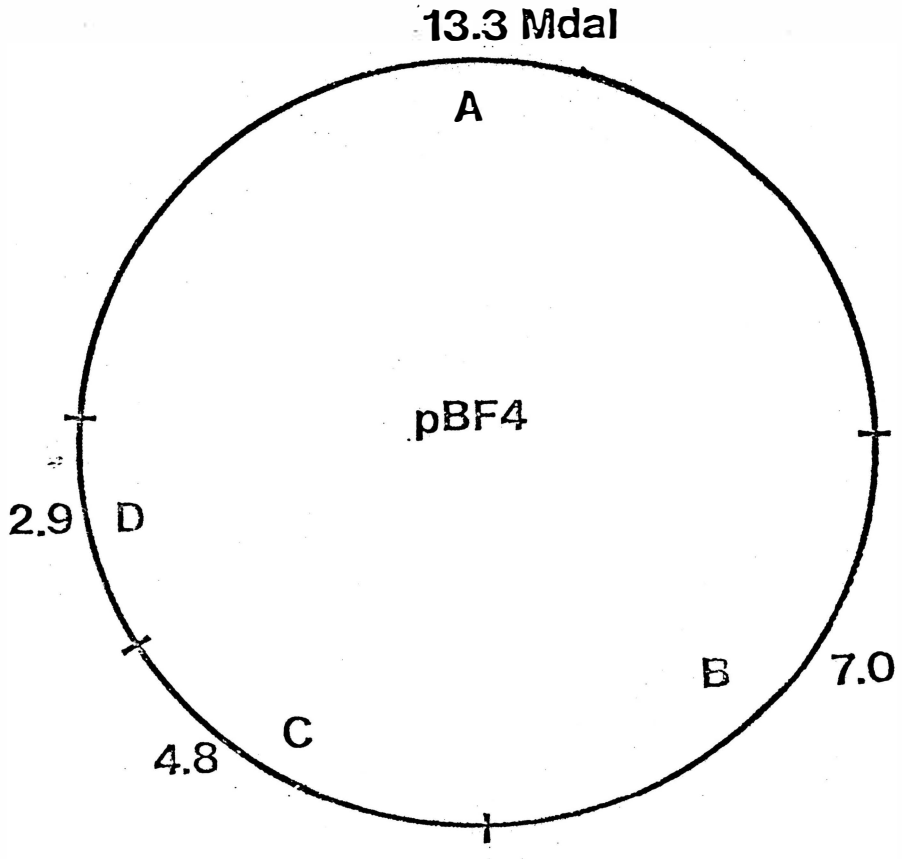
(a) Based on 8 separate digests
(b) 'doublet species identified by use of scanning densitometer
(c) Based on 7 separate digests

fragments B' plus D being contiguous is ruled out since fragment B' is missing in pBF4ΔLM-1 digests yet the partial fragment b is still observable in pBF4ΔLM-1 Hpa I digests. Figure 10 depicts the circular HpaI fragment map for pBF4 deduced from the information gathered from the two pBF4 deletion plasmids and partial Hpa I digest fragments observed with these plasmids. Some portion of the transferable lincosamide-macrolide resistance gene would appear to be located on the Hpa I B fragment since the deletion of pBF4ΔLM2 is limited to just this fragment.

Figure 10

Hpa I restriction endonuclease

fragment map of pBF4



Hpa I fragment map

An ordering of the EcoRI fragments of pBF4 was accomplished by use of partial digests and simultaneous double digestions (hereafter referred to as double digestions) with Ava I. EcoRI fragments B, C and E were ordered by estimating the sizes of the partial digest fragments of EcoRI digested pBF4ΔLM-1. The partial digest fragments a and b were estimated to be 8.6 and 6.9 Mdal respectively. Fragments B plus C give a total of 7.9 Mdal, B plus E give a total of 7.2 Mdal and C plus E totals 4.9. The size of the partial digests can only be explained if the order of fragments is C, B and E. Partial digest fragment a would represent fragment B plus C and partial digest fragment b would be fragments B and E.

This leaves fragments A, D and F to be ordered. Assuming a single deletion event gave rise to pBF4ΔLM-1, fragments D and E must be contiguous with one another and not split between fragment A. Thus, the fragment order to be differentiated is ADF or AFD. Ava I digestion of pBF4 yields a 2.4 Mdal fragment and 24.8 Mdal fragment. Ava I digestion of either pBF4 deletion plasmids reveals that one Ava I cleavage site is lost since only a single large fragment is detectable in Ava I digest of pBF4ΔLM-1 or pBF4ΔLM-2.

In double digests of pBF4 with EcoRI and Ava I, all of the EcoRI fragments are observable except for fragment D. The only new fragment observed is 2.0 Mdal in size and hence this new fragment must have an EcoRI cleavage site at one end and an Ava I cleavage site at the other. In double Ava I and EcoRI digests of pBF4, the 2.4 Mdal distance between

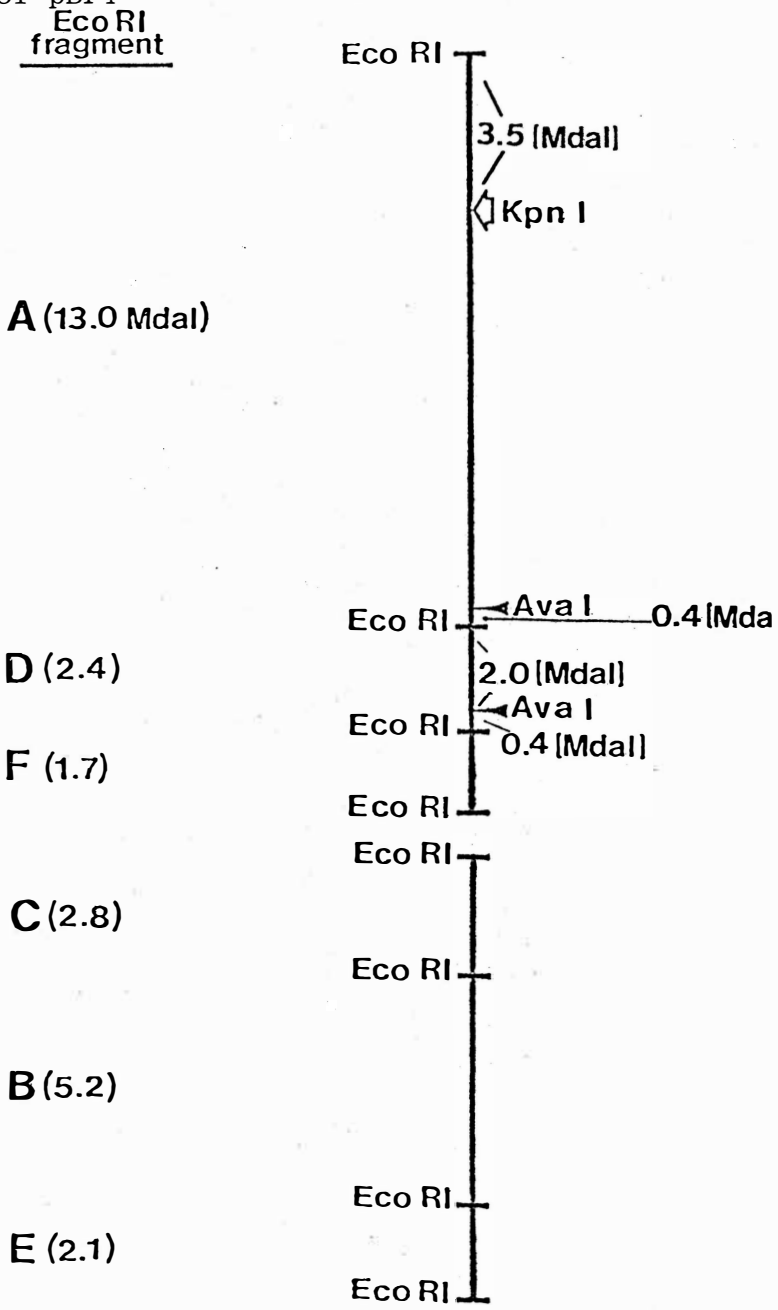
Ava I sites (one Ava I cleavage site must be in the EcoRI D fragment) must span fragments A and D since Ava I digestion into any other EcoRI fragments would easily be detected. From these results the EcoRI fragment order is concluded to be ADF. The right hand Ava I cleavage site is believed to be 0.4 Mdal from the EcoRI cleavage site between fragments D and F. If it were located 0.4 Mdal from the A and D EcoRI cleavage site nearly 2.0 Mdal of the EcoRI A fragment would be cleaved and a 11.0 Mdal fragment in double Ava I and EcoRI digests would be observed. A fragment of this size is not detectable in double digests of pBF4.

Figure 11 depicts the ordering of the EcoRI fragments of pBF4. A complete circular map of the EcoRI fragments has not been possible, therefore the total fragment order could be A, D, F, C, B and E or A, D, F, E, B and C. Double EcoRI and Kpn I digests reveal that the single Kpn I site is 3.5 Mdal from the left hand end of the A fragment.

Thus, there is compelling evidence that some portion of the lincosamide-macrolide resistance gene of pBF4 may be located between the left hand Ava I cleavage site and right hand EcoRI cleavage site of fragment D since this is the approximate span of the deletion for pBF4ΔLM-2.

Figure 11

EcoRI, Ava I and Kpn I restriction endonuclease map of pBF4



It is clear from the Hind III digests of pBF4 and pBF5 that the 32 Mdal DNA insertion occurs within the Hind III D fragment of pBF4. However, the EcoRI digest patterns for pBF4 and pBF5 are equivocal. It is clear that the EcoRI D fragment is missing in the pBF5 digests but whether or not the pBF4 A fragment is represented by either the pBF5 A' and A" fragments is questionable. Hence, EcoRI fragment mapping of pBF5 presents difficulties and this fact will be brought up in the discussion.

Lincosamide-macrolide resistance expression

A review of the literature concerning transferable lincosamide-macrolide resistance in bacterial genera revealed that the expression of lincosamide-macrolide resistance can be either a constitutive or an erythromycin-inducible phenomenon. Therefore, we wanted to establish the nature of the expression in Bacteroides strains harboring the transferable lincosamide-macrolide resistance plasmid pBF4. The experimental approach employed was to determine the effect of pre-exposure to low concentrations of erythromycin on the growth behavior of liquid cultures challenged with high concentrations of erythromycin. Figure 12 is a graphical representation of the growth response of V544 (pBF4) measured in units of optical density versus time. Figure 12 is representative of the growth responses seen in two separate experiments using V544 and a similar response was seen in a single expreiment with V479-1. The rate of growth as judged by the

increase in optical density for cultures challenged with 500 μg/ml of erythromycin appears to be similar to the rate for unchallenged cultures whether or not the culture has been pre-exposed to erythromycin. Thus, it appears that the erythromycin resistance gene is constitutively expressed in Bacteroides strains bearing pBR4.

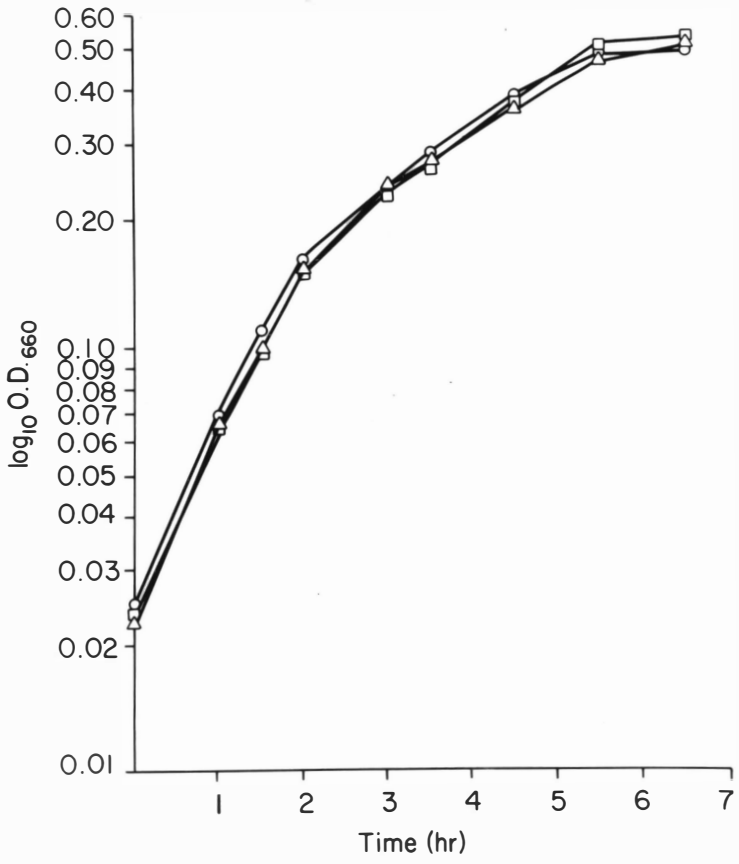
Figure legend

Figure 12. Growth response of V544 in cultures containing erythromycin.

(O———O) overnight inoculum without erythromycin inoculated at 20-fold dilution into PHC broth without erythromycin. (□———□) overnight inoculum without erythromycin inoculated at 20-fold dilution into PHC containing 500 µg/ml erythromycin. (Δ———Δ) overnight inoculum grown in presence of 5 µg/ml erythromycin used to inoculate PHC containing 500 µg/ml erythromycin. Optical density was measured using a Bausch and Lomb Spectronic 20 at a wavelength setting of 660 nm.

Figure 12

Growth response of V544 in cultures containing erythromycin



Tetracycline resistance expression: growth response studies.

Experiments were performed to study the expression of tetracycline resistance. Besides employing strains harboring the transferable tetracycline resistance plasmid pBF5, V479-1 was utilized since in M.I.C. testing it consistently gave rise to a few colonies on PHC agar plates containing 10 µg/ml tetracycline.

Figure 13 shows the growth response as measured by optical density units versus time of PHC broth cultures for three different strains. The top panel (A) shows the growth response of V479-1 which contains the transferable lincosamide-macrolide resistance plasmid pBF4. As previously noted (Table 5), V479-1 has a tetracycline resistance phenotype of <10µg/ml. Panel (B) depicts the growth response of V599, a spontaneously occurring plasmidless derivative of V479-1. The bottom panel (C) shows the growth response for V514 which has the transferable lincosamide-macrolide and tetracycline resistance plasmid pBF5.

For each strain, the circles represent the case where cells grown in the absence of tetracycline are inoculated into antibiotic-free PHC. The squares in each panel represent the case where the overnight inocula grown in the absence of tetracycline are introduced into flasks containing tetracycline at a concentration of 5 µg/ml. The triangles show the response of PHC cultures containing 5 µg/ml but which were inoculated with overnight cultures grown in the presence of tetracycline at the subinbitory concentra-

tion of 0.1 μg/ml. It is clearly seen in this figure that the growth responses under these conditions for V479-1 and V599 differ from V514. It should be noted that in similar experiments involving the tetracycline resistant progeny of V514, V601 shows the same response as V514 even at a challenge dose of 20 μg/ml of tetracycline.

Thus, the growth response studies for expression of tetracycline resistance reveals that there were apparently two qualitatively different tetracycline resistance phenotypes observable. For V479-1 and V599 there is an inducible tetracycline resistance phenotype and for V514 the tetracycline resistance is constitutively expressed.

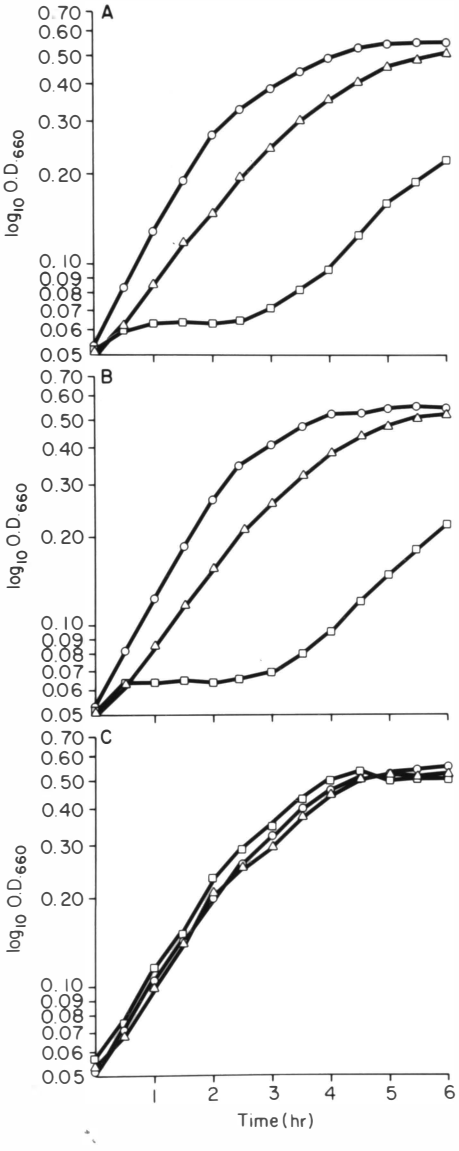
Figure legend

Figure 13. Growth response of V479-1, V599 and V514 in cultures containing tetracycline.

Panel A shows the growth response for V479-1. Panel B shows the growth response of V599 and Panel C depicts the response for V514. In each panel, (O———O) represents the case where an overnight culture without tetracycline is inoculated at a 20-fold dilution into non-antibiotic containing PHC broth. The (□———□) symbol represents the case where the same inoculum is put into PHC containing 5 µg/ml of tetracycline. (Δ———Δ) represents the case where the overnight inoculum is grown in the presence of 0.1 µg/ml of tetracycline and then used to inoculate PHC containing 5 µg/ml of tetracycline. Optical density was measured using a Bausch and Lomb Spectronic 20 at 660 nm.

Figure 13

Growth response of V479-1, V599 and V514 in cultures containing tetracycline



Tetracycline resistance expression: efficiency of plating studies.

The growth response studies for expression of tetracycline resistance revealed that there were apparently two qualitatively different tetracycline resistance phenotypes observable. V479-1 and V599 demonstrated a clear resistance to 5 μg/ml of tetracycline if these strains were preexposed to tetracycline. V514 on the other hand, displayed resistance to concentrations as high as 20 μg/ml whether or not it had been preexposed to tetracycline. Additional studies using a different experimental approach were performed in order to clarify these resistance phenotypes and to determine more clearly the genetic location of the tetracycline resistance determinants. The method employed simply involved growing various Bacteroides strains in overnight broth cultures with and without 1 μg/ml of tetracycline. The overnight cultures then were appropriately diluted and approximately 1000 colony forming units were spread onto plain PHC agar plates and PHC agar plates containing 10, 20 and 50 μg/ml of tetracycline. These plates then were incubated and the number of colonies read after 48 hours. The efficiency of plating is expressed as the number of tetracycline resistant colonies divided by the number of colonies appearing on plain PHC times 100. The results of this approach for a number of key strains isolated in this study can be seen in Table 19. As was the case in growth response experiments, strains V479-1 and V599 demonstrated

Table 19

Expression of tetracycline resistance: efficiency of plating (E.O.P.)

strain	*Bacteroides* species	plasmid/size (Mdal)	transferable resistance	culture grown without or with tetracycline (1 µg/ml)	Tc^r CFU/total CFU (x100) tetracycline concentrations 10 µg/ml	20µg/ml	50µg/ml
V479-1	*fragilis*	pBF4 / 27.2	LM	-	0.1	0	0
				+	100	0	0
V599	*fragilis*	LM sensitive variant of V479-1, plasmid-free	—	-	0.1	0	0
				+	100	0	0
V514	*fragilis*	pBF5 / 60	LM, Tc	-	100	100	0
				+	100	100	0
V537	*fragilis*	pBF5ΔLM-1 /53	Tc	-	100	100	0
				+	100	100	0
V691	*fragilis*	pBF5ΔLM-3/35.5	-	-	0.1	0	0
				+	100	0	0
V528	*uniformis*	plasmid-free	-	-	0	0	0
V544	*uniformis*	pBF4 / 27.2	LM	-	0	0	0
V601	*uniformis*	pBF5 / 60	LM, Tc	-	100	100	0
				+	100	100	0

a clear tetracycline resistance phenotype only when they were exposed to tetracycline prior to plating on agar media containing 10 µg/ml of tetracycline. Strain V514 on the other hand displayed a higher tetracycline resistance phenotype and this higher resistance occurred whether or not V514 was pregrown in the presence of tetracycline. This same resistance phenotype was observed for strain V537 but not for V691. Both strains are lincosamide-macrolide sensitive variants of V514 but V691 has undergone a much larger plasmid deletion than V537. V691 displays the same tetracycline resistance phenotype as V479-1 and V599. Additionally, V537 still demonstrates a transferable tetracycline resistance whereas V691 does not.

When the standard recipient strain V528 is examined using this protocol, the overnight subculture containing 1 µg/ml of tetracycline will not even grow. The lincosamide-macrolide resistant B. uniformis progeny of the V479-1 and V528 mating (V544) also will not grow in the subculture containing 1 µg/ml of tetracycline. V601 which is the B. uniformis lincosamide--macrolide and tetracycline resistant progeny of the V514 x V528 cross demonstrates the same tetracycline resistance behavior as V514. Thus, there appears to be positive evidence for the linkage of the constitutive tetracycline resistance to pBF5.

Expression of tetracycline resistance transfer.

Sebald's group has reported that B. fragilis strain 92 displays a tetracycline-inducible resistance similar to the expression we have observed for V479-1 (84). Additionally,

she has published that the transfer of tetracycline resistance by strain 92 occurs only after growth in the presence of tetracycline. Hence we attempted to examine if V479-1 coul be similarly induced by tetracycline to transfer tetracycline resistance. V514 was excluded from this study since the plasmid-mediated constitutively expressed high level tetracycline resistance phenotype seen by V514 is not the same tetracycline resistance phenotype which had been observed for strain 92. V479-1 was grown in a series of three overnight broth cultures, containing 0.5 µg/ml tetracycline and after each overnight incubation an aliquot was removed for use in a 24 hour mating with the standard recipient V528. A parallel subculture routine and mating procedure was carried out for V479-1 but with tetracycline absent from the cultures. Following the matings, progeny were scored for transfer of clindamycin resistance above and tetracycline resistance alone. The frequency of transfer of clindamycin resistance remained within the 10^{-6} range for clindamycin resistant progeny per input donor cell in each of three successive matings for both the tetracycline exposed and non-exposed V479-1 cells. There was no transfer of tetracycline resistance observed for V479-1 treated in any fashion. This experiment was repeated in a second instance where the tetracycline concentration in the overnight cultures was increased to 1µg/ml and still V479-1 was not observed to transfer tetracycline resistance.

DISCUSSION

At the time this research was initiated there was clear evidence for the existence of extrachromosomal DNA or plasmids in members of the Bacteroides genus. However, at that point no phenotypic trait had been shown to be plasmid associated. In fact, no means of genetic transfer of phenotypes had been demonstrated to occur in Bacteroides. Research interest in the genetics and plasmid biology of this group of organisms developed in the early 1970's when it became apparent that Bacteroides fragilis was a significant opportunistic pathogen and a member of a genus of bacteria found far more prevalent in the human microflora than ever previously thought. Additionally, it was discovered that members of the Bacteroides genus were inherently resistant to a wide range of antimicrobials. Therefore, it was hypothesized that Bacteroides may serve as a source of antibiotic resistance genes and/or reservior of resistance plasmids.

Lincosamide resistance is an unusual and clinically significant property in Bacteroides fragilis. The report that strain 92 from Sebald's laboratory was lincosamide resistant prompted the genetic and plasmid-related studies related in this investigation. Upon receipt of this strain which was renamed V479, the strain was identified as Bacteroides fragilis based on simple phenotypic criteria outlined in the VPI Anaerobe Manual. It had been reported to be resistant to clindamycin and erythromycin. M.I.C. test-

ing with these antibiotics confirmed Sebald's findings. However, she had reported that this strain was tetracycline resistant. When approximately 1,000 colony forming units of this strain were spread onto agar plates containing 10 µg/ml of tetracycline only a few (< 10) colonies would appear after 48 hours of incubation. Therefore it was judged that V479 as a population of cells had a M.I.C. for tetracycline less that 10 µg/ml and thus could not be described as tetracycline resistant in phenotypic terms. One of the colonies from the V479 M.I.C. testing which did appear on the 10 µg/ml tetracycline plate was streaked onto a PHC agar plate containing 25 µg/ml of tetracycline. This clone (V514) readily grew on plates containing 25 µg/ml of tetracycline and demonstrated a definite tetracycline resistance phenotype in M.I.C. testing. V514 also retained the high level lincosamide-macrolide resistance phenotype of its parental strain.

The high level lincosamide-macrolide resistance phenotypes for strains V479-1 (a single colony isolate of V479) and V514 prompted an investigation of the ability of those strains to transfer the antibiotic resistances to lincosamide-macrolide sensitive intestinal Bacteroides strains. Spontaneously occurring rifampicin resistant derivatives of several lincosamide-macrolide sensitive intestinal Bacteroides strains were isolated and used as prospective recipients of lincosamide-macrolide resistance transfer in filter matings with V479-1 and V514. The filter mating technique was chosen since the donor and recipient cells are main-

tained in close contact with one another and the interference of fluid movement in broth cultures is avoided. Therefore, the filter mating technique provided the best chance of observing a genetic transfer event that might occur at a low frequency. Stable lincosamide-macrolide resistant progeny were obtained in these matings using either of the donors. When the direct selection was for erythromycin resistant progeny alone, these progeny were always found to be clindamycin resistant as well. The converse was also found to be true. Thus, this was the first suggestion that the single enzymatic mechanism resulting in cross resistance to lincosamide and macrolide families of antibiotics which is commonly seen in other bacteria may be taking place with Bacteroides fragilis strains V479-1 and V514 (25). The frequency at which either donor strain tranfers lincosamide-macrolide resistance are comparable and the 10^{-6} - 10^{-7} frequency itself represents a frequency range comparable to other naturally occurring resistance transfer systems. The discovery of transferable lincosamide-macrolide resistance in B. fragilis represents the most clinically relevant finding in this investigation since clindamycin is the antibiotic of choice for use in infections involving this organism. The demonstration that V514 is a donor of tetracycline resistance is of less clinical interest since that drug is rarely used in Bacteroides infections. However, further studies on the genetics and expression of tetracycline resistance in V479-1 and V514 were undertaken since they revealed that recombi-

national events were clearly occurring with the strains. It should be noted that the bulk of the experiments involving the basic biological characterization of the genetic transfer event were undertaken with the transfer of the clinically relevant lincosamide-macrolide resistance phenotype by V479-1.

The lincosamide-macrolide transfer process by V479-1 proved to be insensitive to DNase treatment, ruling out transformation as the mode of genetic exchange. Donor cell filtrates or chloroform-treated cell supernatants were not able to mediate the resistance transfer. These facts argue against a transductional (bacteriophage-mediated) event. The lincosamide-macrolide resistance transfer was prevented when the donor and recipient cells were separated by membrane filters. All of these facts taken together imply that a conjugal-like event results in the transfer of resistances. The clear dependence on cell-to-cell contact between donor and recipient is a very strong arguement in favor of this conclusion based on the classic definition of conjugation put forth by Davis (21). However, the fact that the transfer event is not detectable in mixed broth cultures of the donor and recipient cells indicates that there is a qualitative difference between the conjugal event in the enteric bacteria and the genetic transfer event in Bacteroides. Clarification of these differences await experiments which demonstrate the absence or presence of sex pili and the identification of genes responsible for the transfer phenotype.

The limited survey of Bacteroides recipients reveals that there is probably no species barrier among the intestinal Bacteroides to lincosamide-macrolide resistance transfer by V479-1. However, finding a detectable recipient of lincosamide-macrolide resistance is a strain-specific phenomenon. Although no strain of "3452-A" tested proved to be a detectable recipient it is no more unrelated to the fragilis species by the criterion of G+C mole fraction than the ovatus species which is a detectable recipient. Likewise, no distasonis strain appeared to be a recipient but a uniformis strain with a greater difference in G+C ratio from the fragilis species proved to be a recipient. Therefore, if a large battery of intestinal Bacteroides strains were available the results of this survey would predict that a suitable recipient strain would be found for each of the members of the intestinal DNA-DNA homology groups. This statement is further supported by the knowledge that among all of the DNA-DNA homology groups, B. uniformis strains have the least average DNA-DNA homology with B. fragilis 2553 (17%) and all of the other homology groupings fall within the 17 to 90% range with B. fragilis 2553 (47). It is further suggested by these in vitro studies that the in vivo transfer of this lincosamide-macrolide resistance gene could lead to a dissemination of the resistance gene to species far more prevalent in the normal intestinal flora than B. fragilis. Thus, the distinct possibility exists that the lincosamide-macrolide resistance gene may come to

reside in the gene pool of organisms which comprise the bulk of our normal intestinal microflora.

Another biological parameter of lincosamide-macrolide resistance transfer was the investigation of the kinetics of the resistance transfer. The results of the kinetic transfer experiments involving the isogenic pair V545 and V532 indicate that the resistance transfer begins within two hours of mixing donor and recipients and is essentially complete within four hours. Precise evaluation of the early events in the resistance transfer are limited by use of the Gas-Pak system which cannot produce immediate and thorough conditions of anaerobiosis. Experiments in this regard would be possible with an anaerobic glove-box and would probably result in the detection of transfer at an earlier time.

The greatly enhanced transfer frequency seen in the isogenic matings ($\sim 10^{-3}$) as compared to interspecies matings ($\sim 10^{-6}$) suggests the presence of a host restriction-modification system in the Bacteroides genus. However, this is speculation and would require further testing. For example, the observed differences in transfer might be due in part to interstrain differences in surface receptors needed for transmission or differences in the ability to express the lincosamide-macrolide resistance.

The observation that V479-1 and V514 could serve as donors of lincosamide-macrolide resistance combined with the evidence that the transfer event was conjugal-like led to the examination of those strains and their drug resistant

progeny for plasmid DNA. The drug resistant progeny of V479-1 were seen to harbor a plasmid equal in size to the single plasmid species seen in the original donor. The association of the plasmid (pBF4) with the lincosamidemacrolide resistance phenotype is further strengthened by the demonstration of a plasmid similar in size in the drug resistant progeny in the secondary crosses of out of the B. uniformis background (Cross 2, Table 5). The demonstration of plasmid transfer in the secondary matings also suggests that the genes responsible for the conjugal-like transfer are located on the pBF4 plasmid and not located on the V479-1 chromosome, since the pBF4 plasmid is transferred out of a genetic background (V528) which presumably does not possess any transfer genes.

Of additional interest was the observation that V479-1 and V514 harbored single plasmid species of differing sizes. V514 harbors pBF5 and a plasmid equal in size to this plasmid is seen in the lincosamide-macrolide and tetracycline resistant progeny of V514. Hence, it appears that pBF5 which is bigger than pBF4, mediates transferable resistance to tetracycline as well as lincosamide-macrolide antibiotics.

A thorough investigation of the molecular size of the two plasmids using estimates based on three different physical criteria is presented in Table 10. Contour length measurements are usually taken to be the best estimates of a plasmid size next to actual sequence analysis. However, this investigation shows that electrophoretic migration of either the CCC DNA or linear DNA fragments in agarose gels can produce molecular size estimates in very close agreement

to the contour length measurements. In fact, the size differences between each of the methods are less than the standard deviation seen for the contour length measurement.

Besides the physical parameter of size for pBF4 and pBF5, an investigation of the number of copies of each plasmid species per chromosomal equivalent revealed that both plasmids were of low copy number. This is not an unusual finding when compared to the plasmid systems of other bacteria where plasmids in the size range of pBF4 and pBF5 are rarely seen to occur in multiple copies per genome equivalent. (25).

It was of interest to elucidate the physical relationship between pBF4 and pBF5. Prior to the discovery of site specific restriction endonucleases this relationship would have been probed with DNA-DNA hybridization technology and electron microscopy of heteroduplex molecules. However, the use of a battery of restriction endonucleases enables a similar study to be performed with much less time and effort. With this rationale in mind, several restriction endonucleases each with different cleavage sites were used to digest the pBF4 and pBF5 plasmids. The resulting fragments were separated on the basis of molecular size by electrophoresis in agarose slab gels. Each of the fragment patterns resulting from enzymes with multiple cleavage sites led to a similar conclusion. Usually all but a single fragment of pBF4 had a sister fragment of equal size in the pBF5 digest. Thus it appears that pBF5 resulted from the insertion of approxi-

mately 32 Mdal sequence of DNA into pBF4 or the possibility exists that the parental plasmid is pBF5 and pBF4 is the result of a 32 Mdal deletion of pBF5. The latter possibility is thought to be unlikely since an examination of the plasmid content in the original parental strain V479 revealed a plasmid equal in size to pBF4. Thus, it appears the unusual tetracycline resistant strain V514 is the result of a recombinational event where a 32 Mdal DNA segment encoding tetracycline resistance came to reside by insertion into a single site on the transferable lincosamide-macrolide resistance plasmid pBF4 which was originally found in V479 (Sebald's strain 92). The origin and biological nature of this tetracycline resistance will be taken up later in the discussion.

The isolation of lincosamide-macrolide sensitive vari of V479-1 and V514 which had undergone selective plasmid loss (curing) was intended to strengthen the association of the resistance phenotypes with the plasmids. The ability to isolate strains harboring plasmid deletions rather than just plasmidless variants of V479-1 and V514 represents a significant finding in this investigation. This discovery lead to the association of the loss of the lincosamide-macrolide resistance phenotype with the loss of a specific restriction endonuclease digest fragment. The lincosamide-macrolide resistance gene in part or possibly in whole therefore appears to reside on the Hpa I B fragment or EcoRI D fragment. This localization of the clinically significant resistance gene will permit future homology studies between the gene found in V479 and

lincosamide-macrolide resistance genes found in other bacteria. Additionally, if recombinant DNA cloning technology becomes applicable with the Bacteroides genus, specific restriction endonuclease fragments harboring a resistance gene are now available for use in vector construction.

There are several conclusions to be drawn from the data gathered on the isolation of lincosamide-macrolide sensitive variants (Tables 12, 13, and 14). First, pBF5 appears to be a physically less stable plasmid than pBF4 since lincosamide-macrolide sensitive variants bearing smaller plasmids are more readily found in the strains harboring pBF5. Based on this difference in physical stability, we submit that pBF4 is the stable ancestral plasmid and pBF5 is the result of an integration event leaving pBF5 in a "deletion prone" state. Such a physical state is very similar to the deletion phenomenon associated with the tetracycline transposon Tn 10 described by Kleckner et al (51). When Tn10 is inserted into a genome, it can often result in deletions bordering either side of the insertion site. Although the tetracycline resistance insertion described in this investigation has not been demonstrated to behave as a transposable element, the high percentage of deletion formation associated with its presence in pBF5 is suggestive that it may be such a genetic element.

Ethidium bromide initially was utilized since it has been employed in the past as a plasmid "curing agent". However, in this investigation, this compound did not signifi-

cantly increase the number of lincosamide-macrolide sensitive derivatives isolated. Instead, the isolation of the drug sensitive clones appears to be a function of whether or nor a selection for resistance genes was maintained. Sub-cultures made over a long period of time in the absence of lincosamide-macrolide antibiotics were found to contain numerous drug sensitive clones as the result of spontaneous deletion of the lincosamide-macrolide resistance gene.

The deletions appear to be heterogenous in size although several independently isolated drug sensitive variants appear to have plasmids very similar in size (V691, V695 and V698). Whether or not these are chance isolates or representative of a specific class of deletions cannot be answered until many more independent drug sensitive variants are isolated. Additionally, a thorough restriction endonuclease fragment analysis combined with heteroduplex studies would be required to measure the length and points of deletion.

The accumulated restriction endonuclease fragment data presented in Tables 15, 16, 17 and 18 represents the first structural analysis of any Bacteroides R plasmids. The object in this portion of the study was to generate a restriction endonuclease fragment map which would be employed in future genetic studies of pBF4 mediated phenotypes (e.g., transfer, plasmid incompatability, etc.). A circular Hpa I fragment map was constructed (Figure 10) as was a partial EcoRI fragment map (Figure 11). Attempts to circularize the EcoRI fragment map by performing double Hpa I and EcoRI

digests were unsuccessful. The largest Hpa I fragment (A) and EcoRI fragment (A) closely overlap one another. This prevents a discernable ordering of the smaller Hpa I and EcoRI fragments since the double digest fragments are all within a narrow size range. Another problem with the double Hpa I and EcoRI digests is that digestion to completion has not been observed. The occurrence of partial digest fragments have provided an additional source of confusion.

The fragment analysis has permitted the localization of some portion of the lincosamide-macrolide resistance gene to an area starting from the left hand Ava I site to the right hand EcoRI site of fragment D. (See Figure 11). This represents the distance of the deletion found to occur with pBF4ΔLM-2. This localization amounts to approximately 2.0 Mdal of DNA and the isolation of additional smaller deletion derivatives may yield a more accurate mapping.

The active deletion phenomenon associated with the pBF5 plasmid and its 32 Mdal tetracycline resistance insertion has another transposon-like aspect which is apparent in the comparison of pBF4 and pBF5 EcoRI digest fragments. From the size analysis of fragment A of pBF4 and A' and A" of pBF5, it is not clear if fragment A' or A" of pBF5 is the size equivalent of A for pBF4. There is no basis for concluding that either A' or A" fragment is fragment A. If one assumes that they do not represent fragment A, there are two scenarios for the generation of pBF5. pBF5 may have resulted from two

separate insertion events since both EcoRI fragments A and D of pBF4 are missing in EcoRI digests of pBF5. On the other hand, the loss of the two EcoRI fragments may have been the result of an insertion very close to the EcoRI site between fragments A and D. At the time of the insertion, a small deletion spanning that EcoRI site may have occurred resulting in the loss of fragments A and D. The Hind III data suggests the latter possibility since only a single Hind III fragment (3.1 Mdal) of pBF4 is missing in the pBF5 Hind III digest. Deletions which occur upon insertion of a transposon have been documented (51,54). Heteroduplex analysis of pBF4 and pBF5 may differentiate which of the two events occurred. However, the relatively large size of the plasmid molecules involved make heteroduplex analysis a difficult technique to apply in this instance.

Since this investigation represents the initial description of plasmid phenotypes for the intestinal Bacteroides group, it was logical to investigate the regulation of these newly discovered phenotypes. In addition to this rationale, the regulation of antibiotic resistance genes has been the focus of numerous interesting studies. In particular there is very good evidence for two different kinds of regulation for the lincosamide-macrolide resistance genes found in staphylococci and streptococci(112). The constitutive-type expression which is common for many of antibiotic resistance genes is demonstrable for some of the lincosamide-macrolide resistance genes. The second observed

manner of lincosamide-macrolide resistance expression is curious. Besides acting as just an inducer for a higher level of erythromycin resistance, erythromycin acts as an inducer of a higher level of resistance expression for lincosamide drugs as well. On the other hand, no lincosamide antibiotic will demonstrate inducing behavior. With all this in mind experiments patterned on published protocols used to study lincosamide-macrolide resistance expression in streptococci were carried out with strains harboring pBF4 (112).

The results represented in Figure 12 suggest that at least macrolide resistance is constitutively expressed in strains harboring pBF4. The apparent growth rate of broth cultures remains unchanged when challenged with high concentrations of erythromycin whether or not they have been pre-exposed to subinhibitory concentrations of erythromycin.

In order to study the expression of tetracycline resistance, the best designed experiments relied on the isolation of the strains listed on Table 19. From the beginning of this investigation the nature or existance of a tetracycline resistance determinant in the V479-1 background was questioned. First, a plasmidless variant of V479-1 was sought since this would permit study of the nature of tetracycline resistance encoded by the chromosome. Different deletion derivatives of pBF5 were sought to permit the localization of the plasmid-mediated transferable resistance.

The growth behavior experiments proved to be telling for V479-1, its plasmidless variant V599 and V514. Cultures of V479-1 and V599 pre-exposed to subinhibitory concentrations of tetracycline continue to grow when challenged with an inhibitory concentration of tetracycline. However, cultures of V479-1 and V599 not pre-grown in the presence of tetracycline demonstrate a completely different growth behavior when challenged with 5 µg/ml of tetracycline (Figure 13). There is an initial lag in the inhibitory effort of tetracycline which is followed by a two hour period where the apparent growth rate is essentially zero. After the two to three hour lag in growth, the cultures begin to show a log phase growth behavior suggesting a de-repression of a tetracycline resistance gene. This inducible tetracycline resistance gene must be located on the chromosome of V479-1 since the plasmidless V599 variant displays the same behavior. The situation for V514 is quite different since the pre-exposure to tetracycline appears to make little difference in its growth behavior when challenged with 5 µg/ml of tetracycline. Hence, the tetracycline resistance phenotype for V514 appears to be constitutively expressed.

The results for tetracycline resistance expression using the efficiency of plating approach were found to be consistant with the growth behavior experiments. The efficiency of plating technique was employed since it enabled the study of a large number of strains in a single experiment. The results shown in Table 19 demonstrate that

the V479-1 chromosome encodes a tetracycline-inducible tetracycline resistance of 10 μg/ml. The V514 genetic background possesses a qualitatively different tetracycline resistance phenotype where there is a constitutively expressed resistance to 20 μg/ml of tetracycline. This particular resistance gene appears to be located on the pBF5 plasmid since a large deletion results in a loss of this phenotype. In addition, the constitutive tetracycline resistance phenotype is co-transferred with pBF5 to the V528 genetic background (strain V601).

The origin of the constitutive tetracycline resistance gene on pBF5 is speculative. It could be totally non-homologous to the chromosomally born gene and could have originated from a completely different source. Perhaps it represents a transposed gene from an an ancestral plasmid or bacteriophage. On the other hand, the gene may represent a case where the V479-1 chromosome and pBF4 plasmid underwent a recombinational event. The chromosomal inducible gene may have been picked up by the pBF4 plasmid and in the recombinational process the repressor binding site lost resulting in a new constitutive tetracycline resistance phenotype.

The inability to reproduce the results on inducibility of tetracycline resistance transfer using V479-1 that Sebald had observed for strain 92 suggests that strain 92 may be a mixed population of cells with differing plasmid backgrounds.

The inducibility phenomenon she observed may have been a selection for cells harboring a pBF5-like background. Additional confusion is introduced when one considers that she reports that the transferable inducible-tetracycline resistance phenotype is associated with a 17 Mdal plasmid in strain 92. However, this plasmid species was identified in a derivative of strain 92 which had been "cured" of lincosamide-macrolide resistance. A plasmid of this size could easily be attributed to a deletion of pBF4. They identify the 17 Mdal plasmid species only in the donor cells of tetracycline resistance and do not demonstrate this plasmid in any tetracycline resistant progeny. We have not observed a 17 Mdal plasmid species in any of our tetracycline resistant progeny.

In conclusion, plasmid-mediated transferable lincosamide-macrolide resistance has been demonstrated in the obligate anaerobic opportunistic pathogen, Bacteroides fragilis V479-1. In addition, two different tetracycline resistance phenotypes have been observed. One is located on the chromosome of V479-1 and the other is located on the transferable lincosamide-macrolide resistance plasmid pBF5. The transferable lincosamide-macrolide resistance plasmid pBF4 has been analyzed structurally using the restriction endonucleases EcoRI, Ava I, Kpn I and Hpa I. From the restriction endonuclease fragment analysis, the clinically significant clindamycin resistance gene has been localized to a 2.0 Mdal restriction endonuclease fragment. Of added significance, this investigation has laid the

necessary groundwork for future genetic studies of *Bacteroides*. Because of this research, it is now possible to carry out experiments on the transfer of chromosomal genes, studies on plasmid replication and the transposable nature of genetic determinants in an obligate anaerobic bacterium.

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