PLASMID DEOXYRIBONUCLEIC ACID OF STRAINS OF THE SACCHAROLYTIC INTESTINAL BACTEROIDES

by

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DEDICATION

To my loving wife, (The Reverend) who more than any other person gave me the courage to prevail over difficult periods and whose love and wisdom were a constant source of warmth and refreshment.

To my parents, and whose longstanding support and love have made this journey through education more bearable and whose encouragement, to follow my interest and curiosity in science, enabled me to withstand when I felt I couldn't.

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INTRODUCTION

The members of the genus *Bacteroides* (Castellani and Chalmers 1919) are Gram negative, non-spore-forming anaerobic rods. These bacilli are non-motile or motile by peritrichous flagella; some strains metabolize carbohydrates or peptone with succinic, lactic, acetic, formic and/or propionic acids as major end products (80).

The saccharolytic, intestinal species comprise a major portion of this genus (13, 81, 175). The members of this group, that are stimulated by bile (commonly called the "fragilis group") were studied using deoxyribonucleic acid (DNA) homology procedures to delineate the genetic interrelatedness of 340 strains (91). Ninety per cent of these strains were found to belong to any one of ten major homology groups or genospecies.

Some species (*Bacteroides vulgatus* and *B. uniformis*) are significant constituents of the human and animal intestinal flora (79, 127, 157). In addition, several species (*B. fragilis* and *B. thetaiotaomicron*) have been commonly found in human clinical specimens from blood cultures, lung abscesses, soft-tissue infections, and infections of the urogenital tract (157, 167). While pure cultures of some strains of *B. fragilis* and *B. thetaiotaomicron* in a semi-solid medium have been shown to produce subcutaneous abscesses in laboratory mice, no pathogenic mechanism has been demonstrated.

Extrachromosomal genetic elements of DNA or plasmids were isolated from four of nine strains of saccharolytic intestinal bacteroides examined. Each strain represented one of the genospecies of the saccharolytic *Bacteroides* (163). Other investigators have also reported

the presence of plasmids in clinical isolates of B. fragilis, B. ochraceus, and in unidentified oral strains of Bacteroides (45, 68, 106, 159).

To date, clindamycin and tetracycline resistance have been the only phenotypic traits assigned with certainty to plasmids of the Bacteroides, all other plasmids are phenotypically cryptic (174).

Systems of genetic exchange or transfer have been studied in detail for some members of the Enterobacteriaceae and Bacillaceae. The relative locations of genetic traits on the chromosomes of these organisms have been mapped. However, little is known of genetic exchange for the anaerobic bacteria, and subsequent mapping of the host genome has not been possible. To date genetic experiments with anaerobic bacteria have consisted of transfection in members of the Bacteroides and conjugation in members of the genera: Clostridium and Bacteroides (26, 27, 47, 48, 115, 106). A recent report has described conjugal transfer of clindamycin resistance from a resistant donor strain of B. fragilis to a sensitive recipient strain of B. fragilis (174). These workers also report the transfer of tetracycline resistance.

In the present study, plasmids in strains of saccharolytic Bacteroides have been investigated. Members of this group of organisms were selected for study because they are found in high numbers in the large intestine of man and animals and are of clinical significance. These specific strains have been well characterized for traits such as: carbohydrate fermentation, metabolic end products, bacteriocin production, cell wall composition, antigenic determinants,

bacteriophage susceptibility, antibiotic susceptibility, and genetic interrelatedness determined by DNA homology studies (80, 12, 17, 90, J. L. Babb. 1977. Ph.D. Dissertation, V.P.I. & S.U., Blacksburg, Va.).

A group of strains was examined in this study for the presence of plasmid DNA. Once located, the plasmid DNA species were then measured for size and were used in plasmid DNA homology experiments. The plasmid DNA homology groups were then compared with the various phenotypic traits to show possible plasmid mediation.

REVIEW OF THE LITERATURE

This review of the literature is separated into the three major areas which underlie this research effort: the taxonomy of the saccharolytic intestinal *Bacteroides* and their relationship to the animal host; the significance of plasmids and the evolution of isolation techniques for them; and DNA homology procedures and their value in determining genetic interrelatedness.

The Taxonomy of the Saccharolytic Intestinal *Bacteroides*

The taxonomy of the microorganisms that Werner described as the saccharolytic intestinal *Bacteroides*, and that have been referred to as the "fragilis group" by other investigators, has been modified several times since Veillon and Zuber assigned the epithet *fragilis* to some clinical isolates of Gram negative rods in 1898 (175, 165). The description by Veillon and Zuber of the organism *Bacillus fragilis* was restricted mainly to its morphological and colonial appearance. The epithet *fragilis* was retained by Castellani and Chalmers in 1919, after reassigning the organism to the non-sporeforming genus *Bacteroides* (29). The genus was enlarged to contain 18 species by Eggerth and Gagnon in 1933 (52).

The taxonomy was further developed by Prevot who reassigned the *Bacteroides* species described by Eggerth and Gagnon to a new genus, *Ristella* (136). Prevot rejected the genus *Bacteroides* as too vaguely described and thus invalid. Beerens in 1963, proposed a new genus, *Eggerthella*, to include those strains whose growth he found to be

stimulated by bile (11). Neither of these genus names has been commonly accepted by American microbiologists.

Holdeman and Moore studied a large number of strains which matched the early descriptions by Castellani and Chalmers (79). They found many of the strains could be arranged into groups corresponding with the species: B. distasonis, B. fragilis, B. ovatus, B. thetaiotaomicron and B. vulgatus. However, many strains shared reactions and the distinction between species was ambiguous. It appeared that a continuum existed among the strains when phenotypic characters such as carbohydrate fermentation, production of indole from tryptophane, and production of catalase were considered. Because of this close similarity, Moore and Holdeman proposed that the groups or species be considered as subspecies of B. fragilis (the type species of the genus) (121).

Clear lines of distinction between groups were shown by Johnson in 1973, in a preliminary study using DNA homologies as a basis for separating the strains (89). A more complete study by Johnson of 340 strains showed that 10 major homology groups existed in the "fragilis group" (90). Thus in 1976, Cato and Johnson proposed the reinstatement of species rank for B. fragilis, B. ovatus, B. distasonis, B. thetaiotaomicron, and B. vulgatus (30). The nomenclature as ammended by Cato and Johnson will be used throughout this treatise.

The Significance of the "B. fragilis group" to the Animal Host

The significance of these microorganisms has only been considered for the past decade. Bacteroides fragilis and related species are

common in the flora of the large intestine of humans and other animals. In several studies, the saccharolytic Bacteroides have been found to be major constituents of the normal flora of the large intestine (58, 120, 122, 157).

It appears that the association between the normal colonic flora and the host is a stable and cooperatively beneficial arrangement. This symbiosus benefits the microorganism in an obvious manner by providing a protected and nutritionally rich niche in which to develop. The benefits to the host are complex and not fully understood. One advantage appears to be the production of vitamins and cofactors by the bacteria which the host can absorb through the intestinal mucosa (101). The normal colonic flora (in large part the saccharolytic Bacteroides) appears to inhibit the instatment of more pathogenic microorganisms such as Salmonella and Shigella (57, 76, 70). In laboratory animals (rat, mouse, and guinea pig) both normal and gnotobiotic, and in in vitro cultures, this inhibition results perhaps from a competition for carbon sources and production of acids and other metabolic end products. However, the conditions that allow for the multiplication of the enteric pathogens in the colon and the onslaught of the disease are unknown.

Several strains of the Bacteroides have been reported to produce bacteriocins in vitro (17, 9, 10, 124). The effects of such bacteriocins on the susceptible members of the normal flora of the large intestine are unknown.

It is thought that bacteriocins which are produced in vivo may be degraded by the extracellular proteolytic enzymes found in the large

intestine (17). What advantage the bacteriocin affords the producing cell is questionable. It appears that the producing cells are outnumbered by the susceptible strains (19, 38, 78, 122). Perhaps bacteriocin production is a laboratory artifact observed only when the producing strains are grown in a nutritionally rich or complex laboratory medium. None of the bacteriocin producing strains examined by Booth et al. (17) produced bacteriocin when grown on a minimal medium.

While the saccharolytic Bacteroides are significant constituents of the normal colonic flora (i.e., quantitatively), one species appears to be isolated clinically in very high frequency: B. fragilis (62). However, B. fragilis constitutes only 0.3-0.6% of the total fecal flora and this compares poorly with B. vulgatus which constitutes 12% and yet B. vulgatus is not commonly isolated from clinical specimens (122). It has been found that the fecal flora accurately represents the colonic flora both quantitatively and qualitatively (120).

The saccharolytic Bacteroides are opportunistic pathogens and cause infections only when the host defense mechanisms are compromised, for example as a break in the mechanical barrier exposing normally sterile areas to normal flora (62). Experimental Bacteroides infections in laboratory animals have been studied to evaluate the effectiveness of antibiotic therapy and to elucidate the pathogenic mechanisms. Several investigators have used mice injected intraperitoneally with pure cultures of B. fragilis (141, 77, 167). This intraperitoneal abscess model has given highly variable results in the degree and percentage of infections. Walker and Wilkins, however,

have found that the results of subcutaneous injections of pure cultures of saccharolytic Bacteroides in a semi-solid medium are very reproducible (167). Using this mouse subcutaneous infection model they obtained preliminary evidence that strains of B. fragilis generally have a high degree of pathogenicity, although there is strain to strain variation. However, the type strains of B. vulgatus, B. distasonis, and B. ovatus, which are less frequently isolated from clinical infections, also produced a high percentage of infections using this subcutaneous abscess model. On the other hand, strains of B. thetaiotaomicron, a species frequently isolated species from clinical specimens, elicited formation of an abscess in less than half the mice inoculated. The relationship between the frequency of clinical isolation and pathogenicity in the animal model is not clear.

The Significance of Plasmid DNA

Extrachromosomal elements of DNA were discovered in the 1950's as the result of observation and experimentation (almost simultaneously) on three major plasmid mediated phenotypic traits: fertility, multiple drug resistance, and bacteriocin production.

Fertility factors. The term plasmid was coined by Lederberg in 1952, to describe, in a generic sense, non-essential extrachromosomal genetic elements (98). The first transferrable plasmid to be identified was the fertility factor (F^+) (178). Lederberg et al. performed a series of matings or genetic crosses with cells of Escherichia coli strain K-12 that were found to be "fertile" or "non-fertile" (99).

From these crosses they concluded that a fertility factor or extra-chromosomal element encodes for the ability to transfer some chromosomal markers from a fertile or male cell to a non-fertile or female cell during conjugation. They also found that this fertility property was infectious (the recipient following the mating was observed to be fertile) and could be eliminated irreversibly upon treatment of a fertile cell with acridine dyes, cobalt, iron, thymine starvation or x-irradiation.

Marmur et al. were the first to demonstrate that plasmids were in fact DNA. They did this by transferring an F'lac$^+$ plasmid (fertility factor containing functional genes of the host lac operon) from a strain of lactose fermenting E. coli to non-lactose fermenting cells of Serratia marcescens (113). Because E. coli and S. marcescens differ in mol % G+C (50% and 58% respectively), DNA's from these cells can be physically separated in a density gradient of cesium chloride during ultracentrifugation. Cells of S. marcescens lacking the F'lac$^+$ plasmid were unable to ferment lactose and demonstrated only one peak in a cesium chloride density gradient corresponding to the host DNA. However, after mixing these cells with plasmid-carrying E. coli cells, they were able to isolate lactose fermenting cells of S. marcescens. These lac$^+$ cells were analyzed and found to contain plasmid DNA having the same density as the E. coli plasmid. The addition of acridine dyes to the lac$^+$ cells gave rise to non-lactose fermenting progeny which were devoid of the second DNA peak corresponding to the E. coli plasmid DNA.

Falkow and Citarella (56) were the first to isolate the fertility factor DNA as an intact entity. Their work supported previous genetic and density gradient observations.

Mutants of F^+ or fertile cells were isolated by Hayes, and Jacob and Wollman. These mutants were found to yield a higher frequency of recombinants than wild type F^+ cells (72, 87). This higher frequency of recombination indicated that more of the host chromosome was being transferred and at a higher rate. Thus these cells were later designated Hfr cells (higher frequency of recombination). Using genetic crosses with Hfr and F^- cells, Jacob and Wollman demonstrated that the host chromosome of the Hfr cells was being transferred by the fertility factor to the recipient or F^- cells. In timed mating experiments using different methods to interrupt the mating, the chromosome of the donor cell could be mapped. By selecting for various phenotypic characters, the frequencies of recombinants arising from the matings were determined and these directly correlated with the genetic loci encoding for the phenotypic characters on the donor chromosome. It was subsequently determined that these Hfr cells or mutants of F^+ cells were in fact cases in which the F^+ plasmid integrated into the host chromosome.

Plasmids have only recently been detected in the anaerobic bacteria and there has been little genetic work reported. Plasmids have been found in Lactobacillus casei and L. coryneformis, Clostridium perfringens, oral isolates of Bacteroides, Bacteroides fragilis and B. ochraceus (33, 85, 45, 163, 159, 68). Recent preliminary work on genetic transfer using anaerobic microorganisms has been reported for

C. perfringens, B. fragilis, B. ochraceus, and B. thetaiotaomicron (149, 105, 114, 174, 69, 27, 16). However, workers describing conjugal transfer in the Bacteroides admit difficulty in reproducing the plasmid transfers and in maintaining stable exconjugates. Most reports of genetic transfer to date have described transferring antibiotic resistance from Bacteroides fragilis (or other species) to E. coli (restriction endonuclease deficient strain). Two research groups, however, have independently reported multiple drug resistance transfer between strains of Bacteroides. Welch and Macrina (174) and Tally et al. (161) have shown transfers of clindamycin and erythromycin resistance from resistant strains of Bacteroides to sensitive strains. Cell-to-cell contact has been found to be necessary for the transfer of this multiple drug resistance. Welch and Macrina have shown that this transfer is insensitive to deoxyribonuclease treatment which would preclude transfer by transformation or transfection. This transfer is not mediated by cell-free filtrates which would indicate that this transfer does not occur by bacteriophage transduction either. These reports of the transfer of multiple-drug resistance in Bacteroides follow, after 20 years, the early work of multiple-drug resistance in the enterics.

Multiple drug resistance. At about the same time that Lederberg was studying fertility, the Japanese were noting an increase in the number of drug resistant strains of Shigellae during epidemics of bacillary dysentery. Transfer of multiple drug resistance in vitro was first described by Ockiai et al. and Akiba et al. (130, 1). In independent studies, they also demonstrated that cell-to-cell contact

was required for the transfer of this resistance. Other investigators were able to transfer this multiple drug resistance *in vivo* in human and animal intestinal tracts using resistant strains of *E. coli* and susceptible strains of *Shigella* (2, 93, 117). Mitsuhashi et al. reported that multiple drug resistance transfer could be performed with substrains of *E. coli* K-12 regardless of the presence or absence of the F factor, indicating that the F factor is not required for successful transfer (118). The possibility that a chromosomal mutation was responsible for such multiple resistance was shown to be an invalid hypothesis for several reasons: i) the probability of spontaneous multiple resistance to several non-related drugs is quite low (116); ii) transfer was carried out regardless of host chromosome involved (170); iii) treatment of multiple-drug-resistant strains with acridine dyes transformed them with low frequency to susceptible strains and with no signs of segregation to individual resistance patterns (171).

Since the early Japanese work, considerable study has been made of the genetics of resistance transfer factors (74). Resistance transfer factors (RTF's) are extrachromosomal elements of DNA which bear genes that encode for self-transfer and multiple resistance to antibiotics or antibacterial drugs (126). RTF's are not limited to the *Enterobacteriaceae*. Multiple drug resistance factors have been reported for other microorganisms such as *clostridia*, and *staphyloccoci* (149, 97).

Production of bacteriocin. Colicinogenesis or bacteriocin production is another important plasmid mediated trait. Gratia in 1932,

described a toxin, produced by a strain of E. coli, which was inhibitory for other strains of E. coli (63). Genetic crosses or matings of E. coli strains performed by Fredericq and Betz-Barean (59) suggested that the colicinogenesis could not be mapped on the host chromosome. Further the ability to produce colicin was transmitted independently, much as the fertility factor is transmitted. Much work on bacteriocin production has been done since these initial studies and the colicin factor has been shown to be similar to the fertility factor in autonomous replication, ability of some factors to integrate into the host chromosome, and the ability for self-transfer (71, 139, 28).

Bacteriocins have been reported in many other microorganisms in addition to the Enterobacteriaceae. Gram positive cells such as Streptococcus faecalis var. zymogenes, Clostridium perfringens, and Staphylococcus aureus have also been observed to have a plasmid-mediated bacteriocin (86, 85, 168). Bacteroides fragilis and related species produce bacteriocin in vitro but this has not as yet been shown to be plasmid-mediated (17). The pneumococci bacteriocin, pneumocin, has been reported to be encoded by genes on the host chromosome (124). This may be an example of plasmid genes integrated into the host chromosome.

The significance of bacteriocin production is still unclear. However, bacteriocinogeny has been associated with pathogenic Staphylococcus aureus phage type 71 and is correlated with virulence in Pasteurella pestis (44, 25). It is unknown whether the genes encoding for these pathogenic traits are borne on the bacteriocin plasmids. E.

coli V was described as a virulent strain for guinea pigs and rabbits because of its "principle V" or unknown factors. H. W. Smith has shown that the increased invasiveness of this strain in chickens correlates with a plasmid-mediated colicin (col V) (155). This plasmid (pCol V^+) is self-transmissable. When it was transferred to a non-invasive strain of E. coli K-12, the non-invasive strain proved to be virulent and caused bacteremias of chickens. It should be noted that pathogenicity could be caused by other gene products encoded on the bacteriocin plasmid and might not necessarily be attributed to the bacteriocin itself. There are better examples, however, of plasmids which directly mediate pathogenicity.

Enteropathogenic E. coli (EEC) has been found to possess two phenotypic traits which are plasmid mediated and which in synergy cause severe diarrhea in piglets. The two traits: K88 antigen production and enterotoxin production are encoded by genes on separate plasmids (50 Mdal and 60 Mdal respectively) (152, 153).

The bacterium Agrobacterium tumefaciens which is responsible for crown gall tumor formation in plants, harbors a large plasmid (100 to 150 Mdal). Tumor formation has been shown to depend on the interaction of genes from this plasmid with the plant host DNA (35, 148).

In addition to plasmids that have been shown to encode for bacteriocin production (37, 124), resistance to antibiotics and (55, 74, 124, 169, 149) and the ability to perform sexual genetic exchange or fertility (86), plasmids have also been shown to bear genes responsible for pathways of metabolism (92, 152, 131), surface antigens (92, 152, 131), interference with bacteriophage propagation (164),

other traits. Plasmids that have not been correlated with phenotypic traits are referred to as cryptic (126).

Role of Plasmids in Bacterial Evolution. In addition to the phenotypic characteristics that plasmids mediate, the presence of identical plasmids in strains of separate species would strongly suggest genetic transfer of plasmids and could indicate a common source of the origin of the plasmid (3, 71). If this is true as evidence would indicate, plasmids could be considered as agents of adaptation that have common origins and cross between organisms that have little or no chromosomal DNA similarity. In a review article on the significance of extrachromosomal elements (including non-integrated phage and viroid DNA) as agents of evolution, Reanny (138) provides an interesting argument for this case.

The classical or orthodox neo-Darwinian theory of evolution proposes that random mutations result in new phenotypic traits. Through positive selection by environmental pressures, some traits are retained and subsequently inherited by later progeny. However, this concept poses a paradox: three-dimensional proteins which are manifested as phenotypic traits are exposed to environmental selection pressures. For a change to occur in the protein molecule, it must first occur at the genetic level. If there is only one copy of each gene, a harmful mutation would be lethal. Additionally the cell must lose a functioning trait to gain a new one. Reanny suggests multiple gene copies provided by extrachromosomal elements would give the cell a "choice". Environmental pressures would select for the cell exhibiting an advantageous gene product over the cell's competitors. The cell would

not have to lose a gene product encoded by host DNA to gain a new one provided by plasmid or viral DNA. Traits collected in this fashion and encoded on plasmid or viral DNA could become part of the host genome by integration through recombination. The sequence of the nucleotide bases in the DNA is known to convey genetic information upon transcription to the mRNA which in turn directs peptide formation. In addition to the genetic information stored as specific base sequencing, other information may be stored as groups of repeating bases or symetrical sequences. The symmetrical sequences are sequences of bases reading the same either repetitously or inversely. For example, a repetitious sequence could be: ATGC ATGC ATCGC ... The sequence ATGC is repeated several to many times. An inversely repetitious sequence is called a palindrome. A palindrome is a word or sentence that can be read from either direction (much as: "level" or "A man, a plan, a canal panana".) Genetically, a palindromic sequence could be: AAGCTT. Thus with the complementary strand of DNA ready: TTCGAA (172).

Nucleotide symmetrical sequences, in tandem or inversions (palindromes), are known to be involved in regulation as operator sites, as sites of recognition by restriction endonucleases, and as initiation sites for DNA synthesis. Additionally, these symmetrical sequences are known to destablize the DNA in prokaryotes and promote recombination. This duplication or repetition of nucleotide sequences can create mini-DNA's or extrachromosomal elements by regional crossovers between areas of repeating DNA homology. Examples are found in integrating and excisable prophages. Such modular gene regions coupled

with insertion sequences could provide a ready source of gene combinations and flourish under specific selection pressures such as drug therapy. This could be considered a model for the evolution of R factors.

The significance of extrachromosomal elements is presently being examined more critically in light of recent controversy arising from genetic engineering through DNA recombination (43). How or to what extent new techniques in gene transmission and expression will extend is unknown.

Plasmid Structure and the Development of Isolation Techniques

Plasmids are circular molecules of deoxyribonucleic acid and generally exist in the cell as covalently closed circular forms or "ccc" forms (74). This form is characterized by two circular strands of DNA interlocked with one another. The strands are maintained together until a covalent phosphodiester bond is broken. In the entwined state, the molecule twists upon itself into a super helix. This twisting relieves some of the torsional stress of the molecule and produces a more favorable thermodynamic state. Some plasmids exist as an integrated piece of the host genome. Plasmids able to replicate autonomously as well as in this integrated form are called episomes (126). Other plasmid forms, open circular (caused by a break in one of the phosphodiester bonds) and linear, are not thought to exist naturally in the cell unless as a transition form during replication.

The covalently closed form is an interesting molecular species. The following have all been shown to exist in the covalently closed circular form (7): mitochondrial DNA's, chloroplast DNA in Euglena, the kninetoplast DNA in protozoa, the DNA of the papova group of animal viruses, the replicating form of one bacteriophage DNA, several insect viral DNA's as well as the plasmid and episomal DNA in prokaryotes.

Thermodynamically, the ccc form bears special physical and chemical properties. It is thought that the DNA molecules are enzymatically closed to form the ccc configuration with less than the optimum number of duplex turns. The ccc molecule formed in this manner twists upon itself into a superhelix. The formation of the superhelix results in a slightly more stable molecule even though it is more highly organized. While the superhelical form does relieve the molecule of some torsional stress, the remaining stress produces an energetically unstable molecule. Free energy is released upon relaxation of the molecule by a phosphodiester bond break caused by chemical or physical agents. Because of the thermodynamic constraints, the superhelical molecule is restricted in the amount of intercalative dyes it can bind. Such dye binding forces regional unwindings which in turn decrease the number of superhelical turns to produce an energetically favorable condition. However, upon binding dye to the point where no superhelical turns exist, additional dye binding would begin to increase superhelical turns of the opposite sign (or handedness). This is energetically unfavorable and precludes additional dye binding. Linear or open circular forms of DNA are not so restricted

and can bind dye to a saturation point where base-pair steric hindrance would prevent further binding. This binding of dye results in a much greater decrease in the density of linear or open circular DNA than for ccc DNA. This difference in staining allows for the separation of ccc DNA from linear or open circular DNA on density gradients. Additionally, one can separate the ccc DNA from linear or open circular DNA by allowing the DNA molecules to complex with ethidium bromide (or other intercalative dye) and passing the mixture over an hydroxyapatite column. The dye reduces the binding affinity of linear and open circular DNA for the hydroxyapatite to a greater extent than it does for the ccc DNA. For further discussion of thermodynamic properties of ccc DNA, the reader is referred to two review articles (7, 37).

Because of the predominance of the ccc form, many plasmid isolation techniques have been designed to select for this differential staining over the host chromosomal DNA which is fragmented to linear duplex forms during isolation. In early attempts at plasmid isolation, ccc forms were observed but their physical properties were not fully appreciated. One early plasmid isolation technique was designed to separate host DNA from plasmid DNA on the basis of differing mole % G+C ratios and consequently different densities. By transferring a plasmid from E. coli with a mole percent G + C of 50 to cells of Proteus with a mole percent G + C of 38, the plasmid DNA could then be separated from the new host DNA on cesium chloride density gradients during ultracentrifugation (123). The plasmid DNA could be purified further on a column of methylated albumin kieselguhr (MAK). By successively increasing the ionic strength of the eluting buffer, DNA's

of higher molecular weight and lower mole percent G + C were eluted, first. Falkow and Citarella used the MAK column to isolate F DNA of E. coli and found the plasmid to elute quite early (56). Some early workers isolated plasmid DNA using the standard Marmur procedure, which involves lysis of the cells enzymatically or with sodium dodecyl sulfate, deproteinization with chloroform and ethanol precipitation (107). This proved to be too harsh a method because the DNA was fragmented into pieces that were 10 megadaltons (10×10^6 daltons) in size. Helsinki et al. designed a gentler method of DNA extraction (76). In this method, the cells are lysed after spheroplast formation and the lysate is gently extracted with buffered phenol. Residual phenol is then removed by dialysis. DNA so isolated has been found in fragments as large as 100 megadaltons.

An interesting method of plasmid isolation was devised by Inselburg and Levy who used E. coli mutants (K-12, P678-54) called minicells (84, 101). These cells, formed by abnormal cell division, are about one-tenth the volume of normal cells and contain no significant amount of host DNA but still contain enough of the necessary enzymes for DNA replication and genetic expression including metabolism. The mini-cells can be separated quite easily from the normal size parent cells by differential centrifugation. Plasmid DNA can be incorporated into the mini-cells by one of two methods. The plasmid DNA can be transferred to the mini-cells following the conjugation of mini-cells with normal size F cells. The plasmid DNA could also be segreated into the mini-cells following the cell division of parent cells containing plasmid DNA. The plasmid DNA is then isolated from these mini-cells

using any of the standard methods. One advantage in using mini-cells is that the phenotypic expression of the plasmid is easily determined because the plasmids are the only genes present.

An imaginative modification of previous isolation techniques was developed by Freifelder and Freifelder (60). This procedure employs the conjugal transfer of plasmid from a mutant host that is unable to incorporate exogenous radioactively labeled thymine, to a recipient cell that was previously inhibited from replication by heavy doses of ultraviolet light irradiation. Thus following transfer, the plasmid is labeled with thymine and can be detected following its isolation using other conventional techniques.

One of the most difficult problems encountered in plasmid isolation rests with lysing of the cell. Many Gram negative cells are susceptible to lysis with sodium dodecyl sulfate (SDS). Gram positive cells require a partial enzymatic digestion of the outer wall using lysozyme and anionic detergent. Staphyloccoccus aureus requires enzymatic digestion of its outer cell wall by lysostaphin instead of lysozyme (125). Some Gram positive cells such as Streptococcus mutans and Nocardiae require the addition of glycine during logarithmic phase of growth to aid the lysis by anionic detergents (140, 18).

While anionic detergents such as SDS or sodium sarcosinate (Sarkosyl), proteases such as trypsin or pronase, and heat, have been used to lyse many species of bacteria, they also have been shown to cause destruction of plasmid DNA-protein complexes (36). This DNA-protein complex (or relaxation complex) is thought to be a replicative form of the plasmid (94, 37). Clewell and Helsinki

successfully substituted a nonionic detergent polyoxyethylene cetyl ether (Brij 58-Atlas Chemical). They found that the majority of host DNA was bound to the cell membrane and could be sedimented during low speed centrifugation, while plasmid DNA remained in the supernatant (36). Brij 58 appears to bind magnesium ions which causes disorganization of the cell membrane which in turn allows the release of plasmid DNA while chromosomal DNA is still bound.

The current trend in plasmid isolation techniques is to isolate covalently closed circular form. This is important for several reasons: i) not all plasmids contain a self transfer factor or are ammenable to mobilization by another plasmid (37); ii) not all plasmids segregate well into minicells (95); and iii) the majority of plasmid DNA is thought to exist in vivo in many species as the ccc form (95).

One early report of the isolation of plasmid DNA by selecting for the ccc form over the linear fragments of the host was by Radloff, Bauer, and Vinograd (137). Using ethidium bromide, an intercalative dye, to preferentially bind linear DNA in a cesium chloride density gradient, they were able to separate the host DNA of HeLa cells (and open circular DNA) from ccc DNA during ultracentrifugation. When this method is used to isolate plasmids from bacterial cultures, sodium sarcosinate should be used as the lysing detergent because it is more soluble in cesium chloride than sodium dodecyl sulfate (8).

The cleared lysate procedure of Guerry et al. (66) is a short method of plasmid isolation in which the cell is lysed and sodium chloride is added to a final concentration of 6 percent. After overnight at 4 C, the lysate is subjected to moderate centrifugation to

precipitate the host DNA leaving the ccc DNA in the supernatant. Plasmids have been isolated from E. coli, Streptococcus mutans, and "B. fragilis group" using this procedure (66, 140, 163, 68). Other workers have placed cold or radioactively labeled cleared lysates on ethidium bromide-cesium chloride density gradients to isolate plasmids from Pseudomonas aeruginosa, Salmonella typhimurium, and Agrobacterium (65, 164, 179).

One report has described the isolation of plasmids from E. coli using column chromatography on hydroxyapatite with ethidium bromide (132). DNA lysates were prepared by lysis of the cells with Brij 58. The lysate was deproteinized by passage over a column of Sepharose B4. Ethidium bromide was then added to the lysate and the lysate was passed over a column of hydroxyapatite and celite (a column spacing agent). The column was then washed with sodium phosphate-ethidium bromide buffer and plasmid DNA was eluted with a sodium phosphate-ethidium bromide gradient.

The use of adsorbants such as hydroxyapatite or nitrocellulose (cellulose nitrate) for differential binding of double-stranded or single-stranded DNA respectively has been used for plasmid isolation (39, 133). This can be used as a separation technique following the cleared lysate-deproteinization steps already mentioned or following alkali denaturation as described below.

Because of their ccc nature, plasmids remain compact under highly alkaline conditions (pH 12.1 - 12.3) while open circular and linear forms denature and remain single-stranded following neutralization. This technique, first reported to separate the replicative form of

bacteriophage phi X 174 in E. coli, has been used by several investigators in the last three years to isolate plasmids from Pseudomonas putida, Lactobacillus species, and Agrobacterium tumefaciens (88, 133, 33, 42). The procedure described by Currier and Nester couples a brief and gentle shearing of the host DNA from SDS lysates with an alkaline denaturation of the host DNA (42). Single-stranded DNA precipitates when extracted with phenol so it is removed at the phenol deproteinization step. This procedure separates the plasmids of the saccharolytic Bacteroides from the host chromosome more efficiently than the cleared lysate procedure of Guerry et al. (66).

Agarose slab gel electrophoresis has been used widely for the detection of plasmid DNA. Meyers et al. (115) have coupled this technique with the Guerry isolation procedure as a rapid means of surveying for plasmid DNA in E. coli, Haemophilus influenzae, and Neisseria gonorrhoeae. This method of detection is rapid and inexpensive compared with differential ultracentrifugation. Also by measuring the rate of migration of the plasmid DNA species, one can calculate its size from a standard curve.

A modification of the Guerry-Meyers method was recently reported independently by two laboratories (6, 162). Lysates from single colonies of E. coli were sheared on a vortex mixer and subjected to electrophoresis on agarose horizontal gel slabs. Upon staining with ethidium bromide, faint bands were produced. The relative migrations of the bands were determined from photographs of the gels. This procedure is restricted to plasmids that are amplified in the host.

For example, Col El plasmid for which there are usually 20-100 copies per cell, under certain conditions can be amplified to comprise 45% of the total cell DNA and these yield a detectable amount of material.

The Use of DNA Homology Procedures for Detecting Genetic Interrelatedness

The DNA molecule has been called the "master molecule" of the cell, for it contains the genetic code and thus directs the formation of all the cell's phenotypic properties. The flow of genetic information in the cell from the DNA to final peptide assembly is generally well accepted. The specific sequences of nucleotide bases of the DNA molecule hold the cell's genetic information. When a complementary copy of RNA (messenger or m-RNA) is replicated along a portion of one of the strands of the DNA molecule, the genetic information is transferred as specific sequences of the m-RNA. In eukaryotes the m-RNA may be further processed by adenylation or capping before passage from the nucleus to the cytoplasm where it is translated. In prokaryotes less processing occurs and the message is begun to be translated while m-RNA is still being transcribed from the DNA template. Ribosomal RNA (r-RNA) molecules and proteins coassemble on the messenger RNA as ribosomes. As the ribosomes move along the m-RNA, amino acid incorporation into one growing peptide chain is mediated by transfer RNA (t-RNA) molecules. The t-RNA molecules, each specific for an amino acid, transport the amino acid to the ribosome and bind at specific sites. The only t-RNA molecules that can bind are those whose

anticodon (a sequence of three nucleotide bases of the t-RNA) can base-pair with a sequence of three nucleotide bases (codon) of the m-RNA. This specific correlation of anticodon and specific amino acid is the deciphering mechanism of the genetic code. Once the t-RNA is bound to the ribosome, the amino acid being carried is transferred to the growing peptide chain and the t-RNA is discharged. The peptide chain itself is discharged when one of several termination codons is reached by the ribosome on the m-RNA. The process, quite simply described here, results in the formation of peptide chains at the expense of adenosine-5'-triphosphate and guanosine-5'-triphosphate. The peptide chains are able to condense into specific three-dimensional aggregates or proteins. These protein molecules are then used by the cell as structural components, metabolic enzymes, and metabolic substrates which are the phenotypic properties of the cell (99).

One aim of taxonomy in considering bacteria, or any other life form, is to classify them phylogenetically based on their physical appearance and biochemical properties. Because these properties are a partial reflection of their genotype or genetic code, the true identity of the cell rests within the unique nucleotide base pairing sequence of the DNA molecule. Because bacteria unlike many other life forms, lack a confirmed fossil record, evolutionary studies of them are quite difficult. Genetic interrelatedness can be discerned through DNA homology studies. Therefore, DNA-DNA homology and DNA-RNA hybridization studies have been especially helpful in bacterial taxonomy and in elucidating bacterial phylogenetic development (112, 119). By correlating phenotypic characteristics with DNA homology groups, one is

able to determine what traits are characteristic for a given genospecies or homology group. These traits can then be used in classifying other members of the genospecies or homology group (90). DNA-DNA homologies are quite useful for comparing closely related groups of microorganisms, however for studying phylogenetic development of more diverse groups of microorganisms, DNA-RNA hybridization is useful. Ribosomal RNA cistrons are conserved to a greater extent than other cistrons along the DNA and can be hybridized with ribosomal RNA from diverse groups of bacteria to indicate more distinct genetic relatedness (this is well reviewed by Moore) (119).

DNA reassociation kinetics. DNA-DNA reassociation or renaturation involves two single stranded molecules of DNA which collide through random movement and either reanneal or separate. As with any other bimolecular reaction forming a non-interacting product, DNA reassociation follows true second order kinetics. The rate of the reaction is constrained by several factors. The rate of the reaction increases as the temperature decreases below the Tm or thermal melting point (the mid-point of a thermal denaturation curve at which the DNA is 50% denatured) (108). The rate of reaction reaches a maximum at 15 - 30 C below the Tm and then again decreases as the reassociation temperature is decreased further (109). The formation of mismatched base pairs increases as the reaction temperature is decreased further than 25 C below the Tm. This appears as nonspecific base pairing and is detected in decreased thermal stability of the renatured pair (89, 22). The Tm of a particular DNA preparation is a function of the mol % G + C mole ratio, as the mol % G + C increases so does the Tm (108). The mole

fraction of guanine plus cytosine is a useful index of dissimilarity. If DNA's from different strains do not share a similar mole fraction of G + C, then the strains would have little DNA homology with one another. Two methods are commonly used for determining mol % G + C: thermal denaturation profiles and buoyant density ultracentrifugation (106, 147, 110). The reaction rate at the optimum temperature (Tm - 25 C) is dependent upon the ionic strength of the sodium ion below 0.4 M, but is almost independent above this concentration (109). The reaction rate at the optimum temperature is inversely proportional to the solvent viscosity if the solvent viscosity changes the Tm (177). For a constant concentration of a given DNA, the rate of reaction increases with an increase in the molecular weight (108). An increase in redundantcy for a given concentration of DNA also increases the rate of reaction (23).

The rate limiting event under optimum conditions during second order kinetics is the nucleation event or the initial collision with the pairing of a few correct bases (177). The remainder of re-association between the two fragments following the nucleation event must be a fast "zippering-like" reaction where the remaining bases pair in register. If this were not so, the overall reaction rate would appear as first order kinetics. Another effect of viscosity is in producing a hydrodynamic limitation on the "zippering" reaction (177). Because the rate of reaction is a measure of nucleation events, this is thus a rate of collision and is concentration dependent. The product of the DNA concentration and time of incubation is a measure of reassociation. The C_0t value as employed by Britten and Kohne is

a product of moles of nucleotide per liter and time of incubation in seconds (23).

Without digressing into a mathematical treatment, it would be helpful to discuss the basis of the $C_o t$ value from the second order kinetics of DNA reassociation. A decrease in the concentration of single-stranded DNA (C_s) with respect to time (t) is equal to the product of the second order rate constant (k_2) and the concentration of single-stranded DNA squared.

$$\frac{-dC_s}{dt} = k_2 C_s^2 \qquad (1)$$

Rearranging and integrating this equation one obtains:

$$\frac{Cs}{Co} = \frac{1}{k_2 C_o t + 1} \qquad (2)$$

At 50% reassociation, $\frac{Cs}{Co} = \frac{1}{2}$

Rearranging, one finds: $k_2 = \frac{1}{C_o t}$ (3)

where $t_{\frac{1}{2}}$ = the time required for 50% renaturation.

The second order rate constant is therefore equal to the reciprocal of the $C_o t$ value at 50% reassociation. This calculation further supports the observation that DNA reassociation proceeds as a second order kinetic reaction. (For a more complete mathematical treatment, the reader is referred to (176, 177).

The $C_o t$ curve (plotting amount of reassociation against the log of the $C_o t$ value) can provide some helpful information in the form of the genome size. As Britten and Kohne have shown, by plotting the $C_o t$ value of DNA from several different origins, one is able to compare various genome sizes (23). Thus the rate of reassociation is inversely proportional to the complexity of a given DNA (see Figures 7, 8). Initially, these workers were quite surprised to find a fraction of DNA from a complex eukaryote reassociating quickly, with the remainder taking several days to renature. It was suggested, and later supported, that DNA from all complex eukaryotes (plant and animal) contained a portion of repetitive or redundant DNA. There has been speculation that this DNA may serve as genes encoding for important structural proteins (e.g., immunoglobulins), ribosomal RNA and ribosomal proteins, as regulatory genes, and/or as duplication of important genes for many other functions. None the less, the repetitive DNA was discerned by its rapid rate of reassociation uncharacteristic of complex DNA. The $C_o t_{\frac{1}{2}}$ (or $C_o t$ value at the mid-point of the $C_o t$ curve) for mouse satellite DNA (repetitive mouse DNA) is 6×10^{-3} (mole seconds/liter) while the non-repetitive fraction of Calf thymus DNA has a $C_o t_{\frac{1}{2}}$ of 6×10^{4}. The use of the $C_o t$ value at the mid-point of the $C_o t$ curve is a valuable measure of the genome size of the DNA being reassociated.

Bacterial DNA (E. coli), 2.5×10^{9} daltons in size, has a $C_o t_{\frac{1}{2}}$ of 5, while bacteriophage T4 DNA, 1.2×10^{8} daltons in size, has a $C_o t_{\frac{1}{2}}$ of 0.25. Similarly, T4 DNA which is 1/20th the size of E. coli DNA has a $C_o t_{\frac{1}{2}}$ which is also 1/20th that of E. coli. This relationship appears

to hold true for all non-repetitive DNA (of which bacterial and viral DNA are composed). If one knew the $C_o t_{\frac{1}{2}}$ of a given non-repetitive DNA, then one could determine the size of the genome using published standards. While it may seem obvious, the $C_o t$ values serve another important function. Following the linear portion of the $C_o t$ curve near total renaturation, the $C_o t$ value is a good measure of complete reassociation and is used to determine how long a given concentration of DNA is allowed to reassociate to reach completion. Thus in designing a DNA homology experiment, one can calculate the parameters of the experiment (concentration and time of incbuation) given the approximate size of the genome (viral, bacterial, eukaryote, etc.).

In DNA renaturation, the fragments duplex along areas of shared base-pairing. However, very few fragments will align in such a fashion that does not result in some exposed un-duplexed regions. In the S-1 method of analysis these areas of single-strandedness will be degraded into free nucleotides and will not be assayed or detected. Thus the amount of reassociation will always appear lower than that method of hydroxyapatite adsorption. Smith et al. (158) report that the S-1 method of analysis will not yield data reflecting a second order kinetic reaction unless a correction factor is included. These workers observe that single-stranded regions (on fragments also having duplexed regions) will be inhibited from further nucleation events by a factor of 2 to 4. Britten and Kohne (23) using hydroxyapatite to

measure the amount of duplex formation expressed the second order rate reaction in the following form:

$$\frac{C_s}{C_o} = \frac{1}{k_2 C_o t + 1} \qquad (2)$$

However, the second-order reaction assayed with S-1 appears as:

$$\frac{S}{C_o} = \frac{1}{(k_2 C_o t + 1)^n} \qquad (4)$$

where S = amount of S-1 sensitive DNA (single-stranded whether duplexed in part or not) and n = 0.45 (158). When n = 1, equation 4 becomes equation 2 and second order kinetics are followed.

In 1960, Marmur and Lane and Doty et al. reported that double stranded DNA could be denatured and reassociated in vitro (111, 51). Thus began the early DNA duplexing experiments which demonstrated genetic interrelatedness between two different bacterial species (147). Since these early experiments, strains of over 50 different genera or groups have been examined for DNA homology using one of several methodologies (119, 160, 151).

Early DNA duplexing experiments used heavy isotopes with the recovery of hybrid duplexes from isopycnic density gradients (146). McCarthy and Bolton used single-stranded DNA held in agar blocks and reassociated with a radioactive isotope-labeled reference DNA in free solution. The hybrids were eluted from the agar matrix and radioactivity was counted (102). Since these experiments were conducted four major methods have become commonly used. They are: membrane filter, hydroxyapatite, free solution with S-1 endonuclease, and spectrophotometry. Nygaard and Hall allowed DNA to hybridize with RNA

in free solution. Upon filtering this mixture through nitrocellulose filters, they found hybridized RNA would bind to the filters, but free (unbound) RNA would not (129). Gillespie and Spiegelman modified this procedure by allowing RNA to hybridize with DNA bound to nitrocellulose membranes (61). A comparable study was performed by Denhardt, who allowed DNA to renature with DNA bound to nitrocellulose filters. By preincubating these filters (previously bound with single-stranded DNA) in an albumin solution consisting of Ficoll, polyvinylpyrrolidone and bovine albumin, it was observed that non-specific binding of single-stranded DNA was significantly reduced yet specific annealing of denatured DNA to complementary DNA was not affected (49). When these membrane-bound preparations of DNA are incubated in the presence of radioactively labeled DNA (or RNA) under appropriate renaturation conditions, the radioactivity is then counted as an index of duplex formation. This direct binding reaction of DNA with DNA immobilized on nitrocellulose membranes is not a true second order kinetic reaction because the bound DNA is unable to reassociate with itself. A variation of this method approaches a second order kinetic reaction by a competition reaction or indirect binding (82, 91, 81). In this method, the bound DNA and the labeled DNA are from the same strain and are homologous. To this reaction is added a second DNA (heterologous) which is in 100-fold excess concentration to the labeled DNA. The amount of competition by the bound DNA with the heterologous DNA for the labeled DNA indicates any shared homology. This is measured by comparing the radioactivity of the indirect binding of the homologous DNA with the heterologous DNA.

Hydroxyapatite is a calcium phosphate matrix [$Ca_{10}(PO_4)_6(OH)_2$] which can selectively bind single or double stranded DNA depending on the phosphate concentration of the washing buffer (13, 14, 15). Several workers have used the propensity of hydroxyapatite to bind native or duplexed DNA fragments selectively over single-stranded DNA or RNA (20, 21, 24). The double stranded DNA then can be eluted with a higher ionic strength phosphate buffer (0.2 - 0.5 M). Thus by allowing a labeled reference DNA to duplex under appropriate conditions with an unlabeled heterologous DNA then adsorbing to hydroxyapatite, one can wash free any single-stranded DNA (labeled and unlabeled) using 0.12 or 0.14 M phosphate buffer. Upon elution with 0.2 - 0.5 M phosphate buffer, unlabeled DNA duplexed with labeled DNA can be counted for radioactivity and amount of shared homology calculated.

The third major method of determining DNA-DNA homology is a free solution reaction method with S-1 endonuclease. The enzyme belongs to the hydrolase class of enzymes and is considered a class "a" nuclease cleaving the 3' phosphodiester bond (4). The S-1 endonuclease has a pH optimum of 4.4 - 4.6 and requires zinc ions as cofactors for activity. Crossa et al. were the first workers to report the use of this enzyme to determine DNA-DNA homology (39). Labeled reference DNA is first allowed to renature with a large excess of unlabeled DNA in free solution (second order kinetics), the DNA mixture is then incubated with S-1 endonuclease. Single-stranded DNA is degraded mostly to 5' nucleotides and the double-stranded DNA remains intact and can be precipitated with trichloroacetic acid (TCA) and the amount of radioactivity determined. Thus areas along the DNA unable to duplex

because of the low frequency of bases in register are degraded, while regions having a higher degree of paired bases are protected from enzymatic degradation.

A fourth method, not as widely used, to measure DNA-DNA homology is spectrophotometry. When double-stranded DNA denatures, the collapse of the hydrogen bonds allows the separation of strands and results in an increase of absorption at 260 nm. This hyperchromic shift can be measured and is used as an index of native DNA in the sample. The reverse of this phenomenon - a hypochromic shift - can also be measured. Under optimum conditions of renaturation (sodium ion concentration and temperature) heat denatured DNA's are allowed to reanneal. The decrease in absorbance is measured and when the rate of reassociation is compared with rates for the respective homologous renaturations, the amount of shared homology can be calculated (48). Because the rate of reassociation for bacterial DNA (and more complex DNA) is slow, the reaction mixture would require 12-24 hours to reach completion. Such experiments would require the use of the spectrophotometer for long periods of time. Additionally, fewer samples could be measured spectrophotometrically than with other methods. Further discussion of methodologies can be found in several review articles (103, 119).

Plasmid DNA homology studies. DNA homology studies of bacterial host DNA preparations are useful in comparing the similarity of genomes and subsequently correlating particular homology groups with specific phenotypic traits. In plasmid DNA homology studies, one is also able to compare the similarity of different (plasmid) genomes and thus discern any correlation of phenotypic characteristics.

However, an additional advantage may be gained. Because many plasmid DNA species are mobile, one is able to correlate plasmid DNA homology groups with epidemiologic transfer and in many cases determine the origins or foci of such transfer.

There have been many reports of plasmid DNA homology studies within the last seven years.

One of the earliest reports of plasmid DNA homology experiments was made by Silver and Falkow (153). A plasmid DNA species of 65 Mdal (isolated from E. coli) was labeled in vivo using the method of Freifelder and Freifelder (60). Actively growing male cells of E. coli that were unable to incorporate exogenous thymine were mated with female cells that had been irradiated with ultraviolet light to prevent host DNA replication. Tritiated thymine was added to the medium and was incorporated into the R1 plasmid following its transfer into the female cells. DNA reassociation occurred in agar gels as described by Bolton and McCarthy (16). Silver and Falkow were then able to show that the R1 plasmid was 50% homologous with the F plasmid (or sex factor of E. coli), as had been previously suggested by genetic experiments. One of the major contributions of this effort, however, was not provided by the DNA homology study, but by a physical characterization of the R1 plasmid. These workers were able to show, that the R1 plasmid in E. coli appears as a single 65 Mdal entity, but in Proteus mirabilis the R1 plasmid exists as three separate autonomous replicating plasmids of 65, 55, and 12 Mdal. In a subsequent report, Silver and Falkow have demonstrated that the 55 Mdal plasmid of P. mirabilis is the resistance transfer factor (RTF) while the 12 Mdal

species carries the non-self-transferable drug resistance genes (154). This theme of modularity is supported by results of other workers (3, 138, 46).

Guerry and Falkow reported in 1971, that the plasmid DNA homology groups correlated with the plasmid incompatability groups (67). Using radioactively labeled plasmid DNA prepared as previously described, Guerry and Falkow allowed the plasmid to reassociate in free solution and then separated the duplexed DNA from single-stranded DNA on hydroxyapatite (153). These workers were able to demonstrate that several plasmids of the F incompatability group shared a high degree of homology with each other but not with plasmids of the I incompatability group. This work was extended further by Grindley et al., (64) who were able to show that plasmids of the same incompatability group share high homology with each other, but little homology with plasmids of other incompatability groups. Plasmid reassociation experiments were conducted in free solution and subsequently adsorbed and eluted from hydroxyapatite. The plasmids were labeled in vivo and isolated using the cleared lysate procedure of Clewell and Helinski (36). Further research by Anderson et al. (3) and Roussel and Chabbert (142) have shown that resistance transfer plasmids have high homology with members of the same incompatability group regardless of the source of isolation, man or animal. This suggests a common pool of resistance plasmids of the different incompatability groups. These plasmids are able to gain as well as lose. in a modular manner, genes encoding for various drug resistance characteristics. This also suggests that the

evolution of drug resistance is an efficient and progressive phenomenon, with resistance plasmids being selected on the basis of the composition of their resistance genes. These data support the theory of the evolutionary significance of extrachromosomal elements as described by Reanny (138).

Homology studies were conducted by members of the research group directed by Falkow on the ampicillin transposon isolated from strains of Haemophilus influenzae. The ampicillin transposon (TnAp) is a translocational segment of DNA bearing genes for ampicillin resistance that is able to migrate as an intact unit integrating with or excising from a DNA genome (plasmid or host) (126). In a series of three reports, these workers were able to show that several strains of H. influenzae that were resistant to ampicillin, contained a plasmid of either of two sizes (3 or 30 Mdal) (53). With DNA homology techniques, using in vivo labeled plasmid DNA of 5.5 Mdal (containing the TnAp sequence 2.7 Mdal in size) isolated from E. coli and S-1 endonuclease digestion as described by Crossa et al., an homologous sequence equivalent to 1.2 to 1.4 Mdal was seen with the 3 Mdal plasmid and a 2.3 to 2.6 Mdal sequence was seen with the 30 Mdal plasmid (39). These sequences corresponded to the sizes of the structural genes for beta-lactamase and the TnAp translocation sequence respectively. Plasmid DNA homology was thus shown between ampicillin resistance plasmids isolated from H. influenzae and E. coli. Heteroduplex studies confirmed observations from plasmid DNA homology data that suggested that the TnAp sequence was integrated into the 30 Mdal plasmid. One-third

of the transposable TnAp sequence was also found to be integrated into the 3 Mdal plasmid (46).

Elwell et al. (54) found a 31 Mdal plasmid, encoding for tetracycline resistance in H. influenzae, that had 70% base sequence homology with a 30 Mdal ampicillin resistance plasmid isolated from another strain of H. influenzae. The tetracycline resistance plasmid was also found to have 64% homology with a 38 Mdal plasmid that encoded for tetracycline and chloramphenicol resistance in a third strain of H. influenzae. Heteroduplex studies confirmed the presence of homologous regions in the 31 Mdal tetracycline resistance plasmid and the 30 Mdal ampicillin resistance plasmid. The only regions of non-homology appeared to be the TnAp sequence and the tetracycline translocation sequence (TnD). TnD was found as a loop of single-stranded DNA on the end of a duplexed inverted repeated sequence. These workers also demonstrated that the TnD sequence was integrated into the host chromosome of a strain of H. parainfluenzae. The studies suggest that there is a single plasmid existing in the H. influenzae strains with translocating resistance genes, or that there are different plasmids of the same incompatability group each bearing its own set of resistance genes.

Plasmid DNA homology studies were conducted on six plasmid species isolated from strain Y6R of Shigella dysenteriae by Porter et al. (135). These six plasmids were separated on alkaline sucrose gradients as 0.93, 1.1, 2.4, 3.5, 18, and 23 Mdal species. While the 0.93 and 1.1 Mdal plasmids were about 90% homologous with each other, none of the other plasmids share any significant homology. The 3.5

Mdal plasmid has 66% homology with the col El plasmid of E. coli. The 3.5 Mdal plasmid also appears to have some homology with a plasmid-free preparation of host DNA. The extent of homology is represented as 2.4 fractions of the 3.5 Mdal plasmid. These values were determined by using in vivo labeled plasmid DNA binding to unlabeled plasmid DNA fixed on nitrocellulose membranes as described by Denhardt (49). Heteroduplex studies confirmed these plasmid DNA homology values. This strain of S. dysenteriae is immune from the colicin mediated by the Col El plasmid. The 3.5 Mdal species, that has high homology with the col El plasmid, is thought to be a defective col El plasmid with some sequences that are not homologous with col El. However, it is suggested that col El immunity genes are carried on the 3.5 Mdal plasmid and afford the host immunity from the colicin.

Currier and Nester confirmed other reports correlating the presence of large plasmids (107 to 150 Mdal) in Agrobacterium tumefaciens with pathogenicity in plants infected with the host microorganisms (41). In a study comparing 23 plasmid DNA preparations, they found that most plasmids could be assigned to one of two plasmid DNA homology groups. The utilization of the amino acid octopine by the bacteria was characteristic of one plasmid DNA homology group while the utilization of nopaline was characteristic of the other. Later, elegant molecular hybridization work by Chilton et al. (35, 148) was performed showing that genes (associated with tumorogenesis) present on the large plasmids were later inserted into or associated with the plant host DNA through a mechanism as yet undefined.

Crossa, Brenner, and Falkow used the S-1 endonuclease free solution method to compare DNA homology of several plasmids isolated from E. coli (39). This study was conducted primarily to compare the hydroxyapatite method of determining DNA homologies with the S-1 endonuclease method. For bacterial as well as plasmid DNA, the results were quite comparable under optimum conditions.

Rush, Novick, and DeLap used plasmid DNA reassociation kinetics to measure the amount of plasmid DNA isolated from strains of Staphylococcus aureus (145). In a simultaneous study, Novick, Zouzias, and Rush used reassociation kinetics to detect and measure plasmid DNA integrated into the host chromosome encoding for penicillinase (128).

Summary

The saccharolytic intestinal Bacteroides are significant constituents of the normal large intestinal flora of man and animals. Some species have been associated with bacterial infections in man an are agents of acute subcutaneous abscesses in laboratory animals. The pathogenic mechanism(s) of these microorganisms is unknown.

Many strains of saccharolytic Bacteroides have been shown to carry cryptic plasmid DNA. While plasmids in other microorganisms have been shown to encode for many of the host's phenotypic properties including multiple antibiotic resistance and pathogenic antigen production, there as yet is little information on the cryptic plasmids of the Bacteroides.

Homology studies are one means of demonstrating genetic similarity. The presence of similar plasmids in genetically distinct species would

suggest genetic transfer of plasmid DNA from one species to another. Additionally the homology grouping of plasmid DNA could be correlated with phenotypic traits of the hosts. This could suggest plasmid mediation of shared phenotypic characteristics and provide a basis for further studies using genetic techniques to confirm plasmid mediation. Homology studies of plasmid DNA could further show the distribution of particular plasmids throughout the species examined and indicate ecological significance. For example, it would be interesting to note if any plasmid was associated with only abscess isolates, or only fecal isolates, or only isolates from one individual. Such information could again suggest a phenotypic advantage and perhaps suggest a function of the plasmid DNA.

MATERIALS AND METHODS

Materials. All chemicals used were obtained from Fisher Scientific Co. unless otherwise noted.

Bacterial cultures. The Bacteroides strains used in this study are part of the V.P.I. & S.U. Anaerobe Laboratory culture collection and have been characterized extensively by Drs. Holdeman and Moore. The organisms belong to one of 10 major taxonomic groups or genospecies based on DNA homology studies. The strains are listed in Table 1 by their V.P.I. numbers together with other culture collection designations.

The Escherichia coli strains listed in Table 2 were generously supplied by Dr. Stanley Falkow, University of Washington. These strains were tested for purity by determining the Gram stain and by inoculating agar plates to observe any variation of colony morphology.

Growth and Maintenance of bacterial strains. Stock cultures of anaerobic isolates were maintained by periodic transfer under oxygen-free-carbon dioxide into chopped meat broth, prepared as described in the V.P.I. Anaerobe Laboratory Manual (80). Stock cultures of E. coli were stored on refrigerated slants of Nutrient agar (1.5% agar, prepared according to the manufacturer's directions; BBL, Cockeysville, Md.).

Anaerobic cultures for plasmid DNA isolation were grown in tryptone (1.0%) yeast extract (0.25%) broth with glucose (0.6%) supplemented with 1 ml/liter of minimal salts ($CaCl_2$, 0.2%; $MgSO_4$, 0.2%; NaCl, 2.0%; 0.05 M potassium phosphate buffer, and cysteine HCl (0.03%) (TYG) prepared in one liter amounts as described by Cummins and Johnson (40). A culture of each isolate was prepared by inoculating 500 ml

Table 1. Strains of *Bacteroides* used in this study. Source is human unless otherwise noted.[a]

VPI No.	Source and other designations	Isolated from
B. fragilis type I		
2553*	ATCC 25285 Sonnenwirth EN2 NTCC 9343	appendix abscess
2044	CDC 1261	blood
2550	Finegold B11	ischiorectal abscess
3390	Prevot 640-A	?
3625	MCV Bacteriology Lab	uterus
4048	Prevot 2895	sub-phrenic abscess
4082	Prevot 2359	ovarian abscess
4128	Prevot 3020	ascitic fluid
4366	Prevot 2006	septicemia
5001	Lambe 3R665	wound
5650	New Hanover Hospital, Wilmington, N.C. 70-39	myocarditis
6057B	Tuscon Medical Center	buttock drainage
6957	Forsyth Memorial Hospital Winston-Salem, N.C.	blood
11666	Beerens E323	?
12255	Univ. Ill. Hosp. Lab. Chicago, Illinois 9536	blood
12257	Univ. Ill. Hosp. Lab. 11344-1	tissue
B. fragilis type II		
2393	Beerens 12 NCTC 8560	?
2552	Sonnenwirth EN	appendectomy
4076	Prevot 2228	blood
B. fragilis subsp. "a"		
B5-21	VPI	feces
C1-23	VPI	feces
C8-19	VPI	feces
C51-6	VPI	feces
C52-51A	VPI	feces
C54-2	VPI	feces
B. thetaiotaomicron		
5482*	ATCC 29148 NTCC 10582 Werner E50	feces
0489	ATCC 12290	infection following appendectomy
2302	VPI 13M	cecum
3443	VPI	feces
5008	Dalton, MCF	hip drainage

Table 1. Continued

VPI No.	Source and other designations	Isolated from
B. ovatus		
0038-1*	ATCC 8483	feces
2822	Balows (LC) 1-84-7	rectal abscess
3524	VPI	feces
4101	Prevot 1176A	abscess
C11-2	VPI	feces
C40-50A	VPI	feces
C41-35	VPI	feces
C50-15B	VPI	feces
J15-5	VPI	feces
J18-9	VPI	feces
J22-44	VPI	feces
"3452-A"		
2308	VPI 13M	rectal contents
3452-A	VPI	feces
8608	VPI 6460C	ear infection
C10-2	VPI	feces
C14-3	VPI	feces
C15-2	VPI	feces
C27-54	VPI	feces
C51-18	VPI	feces
J7-54	VPI	feces
J15-2	VPI	feces
T1-12	VPI	feces
T1-48	VPI	feces
B. uniformis		
0061-1	ATCC 8492	feces
0909	VPI 2MF92	feces
3537	VPI	feces
3699	VPI	feces
5444A	Prevot 600	?
6387	VPI JSA-6A	cervix
8601	VPI	feces
A7-10A	VPI	feces
A13-47	VPI	feces
B1-15	VPI	feces
C1-10	VPI	feces
C7-17	VPI	feces
C8-30	VPI	feces
C17-3	VPI	feces
C20-25	VPI	feces
C31-12	VPI	feces
C46-14	VPI	feces

Table 1. Continued

VPI No.	Source and other designations	Isolated from
C50-6	VPI	feces
C51-27	VPI	feces
C54-1	VPI	feces
J4-2	VPI	feces
J8-10	VPI	feces
J15-53	VPI	feces
OC22A	VPI	feces
R5-33	VPI	feces
T1-1	VPI	feces
B. vulgatus		
4245*	ATCC 8482	feces
0959-1	VPI 7MF95	feces
2736B	Balows (SJH) 6-756-7	abdominal wound
4506-1	Balows (SJH) I-497-8	abdominal cavity
6186	Baltimore City Hospital Baltimore, MD	pleural fluid
6266A	Dalton, MCV 62875	myocardial abscess
6598B	Corning, N.Y. 1499	wound
C6-7	VPI	feces
C7-2	VPI	feces
C10-6	VPI	feces
C43-46B	VPI	feces
J17-22	VPI	feces
J18-14B	VPI	feces
T1-8	VPI	feces
"T4-1"		
2628	VPI	hog cecum
A19-28B	VPI	feces
B8-5	VPI	feces
C16-2	VPI	feces
C43-5	VPI	feces
C54-23	VPI	feces
J20-53	VPI	feces
R3-5	VPI	feces
T4-1	VPI	feces
B. distasonis		
4243*	ATCC 8503	feces
0052	AEC 9A	mouse feces
A12-1	VPI	feces
B1-20	VPI	feces

Table 1. Continued

VPI No.	Source and other designations	Isolated from
C18-7	VPI	feces
C30-45	VPI	feces
C50-2	VPI	feces
J15-49A	VPI	feces
S1-35	VPI	feces
S6A-50	VPI	feces
T3-25	VPI	feces
B. eggerthii		
B8-51	VPI	feces
C18-8	VPI	feces
C19-14	VPI	feces
C31-22	VPI	feces
S1A-52	VPI	feces
X3-31-1A	VPI	feces
Does not fit any group		
T1-42	VPI	feces

[a]From J. L. Johnson. 1978. Taxonomy of the Bacteroides: I. DNA homologies among *Bacteroides fragilis* and other saccharolytic *Bacteroides* species. Int. J. Syst. Bacteriol. 28:245-256.

*Denotes type or neotype strains.

Table 2. Strains of *E. coli* used in this study

Strain[a] No.	Plasmid No.	Size plasmid (Mdal)
J5	R1	62
J53	Sa	23
C600	RSF1010	5.5
C600	pMB8	1.87

[a]Strain designations are those used by Dr. S. Falkow, University of Washington, Seattle, Washington, who provided these strains.

of TYG under oxygen-free-nitrogen with 8 ml of an 18 - 24 hour anaerobic chopped meat carbohydrate broth culture. The 500 ml culture was then incubated anaerobically at 35 C overnight (18 to 24 hours) which was more than sufficient time for the culture to reach maximum turbidity and achieve late logarithmic or early stationary phase of growth.

Cultures of E. coli were grown for plasmid isolation in a similar manner. An 8 ml aerobic TYG (no cysteine) culture of E. coli was inoculated aerobically and placed on a fast moving reciprocal shaker in a 35 C incubator. After overnight incubation, this culture was used to inoculate 500 ml of aerobic TYG. The 500 ml aerobic TYG culture was placed on a rotary shaker in a 35 C incubator for 18 to 24 hours.

Enrichment of plasmid DNA. Plasmid DNA was enriched using the method of Currier and Nester (42). Cells of bacteria were grown as described and harvested by centrifugation in 500 ml polypropylene bottles at 11,000 x g for 20 minutes in (a Sorvall RC2B High Speed) Refrigerated Centrifuge. The supernatant was discarded and the cell pellet was washed once with 25 ml of Tris-EDTA buffer (TE) (0.05 M Tris buffer (Sigma), 0.02 M ethylenediaminetetraacetic acid, pH 8.0 by the addition of 6N sodium hydroxide). The cell pellet was resuspended in 25 ml of TE buffer, then additional TE buffer was added to a final volume of 200 ml. Sodium dodecyl sulfate (20%) was added to a final concentration of 1%. The cell suspension was then held in a 35 C water bath for 30 minutes to achieve complete lysis.

The lysate was sheared in a 400 ml Sorvall omnimixer cylinder at a setting of 1½ for 3 minutes (or until 5 ml of lysate flowed through a 10 ml pipet with a 1.5 mm tip bore size in 4 seconds; 5 ml of water flowed through in 2 seconds).

The sheared lysate was placed in a 600 ml beaker with a 1½ inch Teflon coated magnetic stir bar. The lysate was stirred at low speed on a multiple stir plate (Lab-Line, Melrose Park, Ill.) while the pH was increased to pH 12.1 - 12.3 by the dropwise addition of 6N sodium hydroxide. The lysate was then stirred and held at pH 12.1 to 12.3 for 10 minutes. Following the alkaline denaturation of host DNA and any nicked plasmid DNA, the pH of the lysate was readjusted to pH 8.5 - 8.9 by the dropwise addition of 6N hydrochloric acid.

Following the decrease in pH, the lysate was stirred for an additional 3 minutes. Dry sodium chloride was then added to give a final concentration of 3%. The lysate was deproteinized by the addition of 50 ml of phenol (Analytic Reagent, grade Mallinckrodt) previously equilibrated with 3% sodium chloride in water. The lysate was stirred for 5 minutes before centrifugation at 11,000 x g for 10 minutes. The aqueous (upper) phase was decanted and any remaining upper phase was removed with an inverted 10 ml pipet. The (lower) phenol phase was discarded. The aqueous phase was extracted with 50 ml of chloroform solution containing 0.3% octanol and stirred at low speed for 5 minutes. The lysate was again subjected to centrifugation at 11,000 x g for 10 minutes.

The aqueous (upper) phase was again decanted and 0.7 volume of cold 95% ethanol was added to the lysate. The lysate-ethanol mixture was stored at -20 C for at least 2 hours--usually, however, it was stored overnight (18 to 24 hours).

The lysate was then subjected to centrifugation at 11,000 x g for 20 minutes and the supernatant was discarded. The precipitate was drained well and then dissolved in 1.5 - 2.0 ml of TES buffer (0.05 M Tris, 0.05 M sodium chloride, 0.005 M EDTA, pH 8.0). This solution was again subjected to centrifugation at 27,000 x g for 20 minutes to remove some polysaccharide, some large RNA complexes, and general debris.

Detection of Plasmid DNA. The concentrated Currier prepared lysates were examined for plasmid DNA by agarose gel electrophoresis. Agarose gel slabs were prepared according to the method of Meyers et al. (115). Agarose (SeaKem) was dissolved in TEB buffer (0.089 M Tris, 0.089 M Boric acid, 0.0025 M EDTA, pH 8.2) to a final concentration of 0.7%. The molten agarose was poured into (glass) gel forms to produce 8.2 cm x 7.0 cm x 0.35 cm gels. Eight sample wells (0.5 cm in width) were formed in the gels by the addition of a "toothed comb" which was inserted into the glass gel form before the agarose solidified. This plexiglass "comb" (7 cm x 1.2 cm x 0.3 cm) was designed with removable brass pins, one in each of the eight teeth. After the agarose gel congealed, the pins were removed to allow air into the sample wells thus eliminating the vacuum that would be created when the comb was removed. While still in the glass gel form, the agarose gel was placed in a vertical electrophoresis chamber (Model GE-4 Pharmacia) filled with TEB buffer. Samples for electrophoresis

were prepared by the addition of 10 µl of dye solution (0.07% bromophenol blue, 7% sodium dodecyl sulfate, 40% sucrose in TE buffer) and 25 µl of concentrated Currier prepared lysate. These solutions were mixed in 500 µl conical polypropylene centrifuge tubes (BioRad) and each total mixture was applied to a sample well on the agarose gel with an Eppendorf pipet. The loaded gel was subjected to a constant voltage of 120 volts, 30 ma for each gel, for 2 minutes, then the voltage was decreased to 60 volts, 15 ma for each gel. The power source was an ISCO - Model 493). Electrophoresis was continued until the tracking dye reached the bottom of the gel slab (about 1-3/4 hours).

Following electrophoresis, the gels were removed from the glass plates and placed in an aqueous ethidium bromide solution (50 µg/ml; Sigma) in small square plexiglass trays for 10 minutes. The ethidium bromide solution was decanted and the gels were rinsed with distilled water. The gels were then viewed in an ultraviolet light view box (ChromatoVue, Ultraviolet Products Inc., San Gabriel, Calif.) and photographed using light of 340 nm on Polaroid 667 film (30 second exposure) with an orange filter (Yoshica 46 mm O-2) and close-up lens (focal length 11 cm). The migration of plasmid and host DNA was measured on the photograph from the bottom of the sample well (origin) to the mid-point of each fluorescent band using a metric ruler (see Figures 1, 2). The molecular weight of plasmid DNA was determined as described by Meyers et al. (115). Plasmid DNA species of previously determined molecular weight were isolated from strains of E. coli using the Currier isolation procedure. A standard curve was constructed on two cycle logarithmic graph paper (see Figure 3). Plasmid DNA R1

of molecular weight 62 x 10^6 daltons (plotted on the ordinate axis) was arbitrarily given a relative migration rate of 10 units (plotted on the abscissa axis). The migration rate of pMB8 plasmid DNA (1.87 x 10^6 daltons) was measured on photographs of agarose gels and compared with the migration rate of R1. A relative migration rate of pMB8 (relatively proportional to the value of 10 units for R1) was calculated to be 35 units. Other points on the standard curve were calculated in the same manner for plasmid DNA's: RSF1010 (5.5 x 10^6 daltons) and Sa (23 x 10^6 daltons). A straight line graph was obtained with a slope of -2.4. Plasmid DNA species of undetermined molecular weights were measured and their relative migration rates were calculated. The graph was then used to estimate their molecular weights.

Further Purification of Plasmid DNA and Preparation of Plasmid DNA for DNA Homology Studies. Purified plasmid DNA was prepared by isopycnic ultracentrifugation in cesium chloride density gradients with the intercalative dye ethidium bromide. Cesium chloride gradients were prepared by mixing 3.4 ml CsCl stock solution (density of 1.90 gm in TES buffer; Apache Chemicals Inc., Seward, Ill.) and 1.75 ml Currier-Nester prepared crude lysate (following centrifugation at 27,000 x g) with 0.25 ml of an aqueous solution of ethidium bromide (10 mg/ml) in 7 ml polycarbonate ultracentrifuge bottles (with noryl cap assembly; Beckman). The gradient tubes were inverted several times to mix the solution thoroughly and were then subjected to a centrifugal force of 97,000 x g in an anglehead type 50 rotor using a Spinco Model L ultracentrifuge (Beckman) at 16 C for 20 hours.

Following centrifugation, the gradients were illuminated under ultraviolet light of 340 nm. The lower fluorescent (plasmid DNA) band was removed using a 16 gauge needle (11.5 cm long) attached to a 10 ml disposable syringe. Multiple preparations were pooled, then all samples were extracted with 1 ml aliquots of isoAmyl alcohol saturated with 6M sodium chloride - 0.6 M sodium citrate, as described by Watson et al. (173). The cleared preparations were then dialyzed against 2 to 3 changes of TES buffer for several hours. Dialysis tubing was prepared by boiling the tubing sections (Fisher Scientific, 20,000 daltons sieve size, 2.4 cm in width) in a 0.5% sodium carbonate solution in distilled water for 5 to 10 minutes.

The extracted and dialyzed plasmid DNA bands from CsCl gradients were then treated with ribonuclease A (RNase). The RNase (bovine pancreatic 0.5 mg/ml, Sigma) was added 50 μl to 1 to 3 ml samples of plasmid DNA and incubated in a 35 C water bath for 30 minutes. Aliquots (1 to 2 ml) of the plasmid DNA samples were then layered onto Agarose gel filtration columns (A 1.5 m BioGel, 1.5 cm x 15 to 17 cm) as described by Currier and Nester (41). The column flow rate was adjusted to 7 ml/hour using a peristaltic pump (The Holter Co.) and degassed 0.1 x SSC (standard saline citrate) buffer (0.015 M sodium chloride, 0.0015 M sodium citrate). Fractions following the void volume and containing material absorbing light of 260 nm were pooled for each sample (see Figure 4). Aliquots (50 μl) for gel electrophoresis analysis were taken from each plasmid sample before and after passage through the agarose columns. Pooled fractions for each sample were passed twice through a French pressure cell (American Instruments Co., Silver

Spring, Md.) at 12,000 psi to obtain fragments of 5 x 10^5 daltons. The sheared plasmid DNA was then dialyzed against 0.1 x SSC then followed by dialysis against distilled water to remove all salts.

The preparations were then freeze dried at -40 C and at a pressure of 150 m of mercury (ThermoVac Industries). The plasmid DNA was then redissolved in buffer [0.02 M sodium chloride, 10^{-3} M N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES)] to a final concentration of 50 to 100 μg/ml. DNA concentration was measured by determining the optical absorbance at 260 nm of a 1 to 60 dilution using 0.-2 M NaCl 10^{-3} M Hepes as diluent. Samples were denatured for 5 minutes in a boiling water bath and were then stored at -20 C.

In Vitro Radioactive Isotope Labeling of Plasmid DNA. Plasmid DNA species from four bacterial strains were selected as reference DNA's for homology studies. The reference plasmid DNA's were isolated in the same manner as other plasmid DNA's with the following exceptions. Following the first CsCl-ethidium bromide isopycnic ultracentrifugation, the plasmid bands from several preparations for each strain were pooled and 5 ml portions were placed in fresh centrifuge tubes for a second ultracentrifugation. Following the second ultracentrifugation, the ethidium bromide was extracted as previously described and the cesium chloride dialyzed away in 0.1 x SSC buffer. The samples were treated with ribonuclease A and then layered on agarose A 1.5 m columns. Plasmid DNA fractions following the void volume were pooled and dialyzed against distilled water. They were lyophilized and then redissolved in buffer (0.02 M sodium chloride, 10^{-3} M HEPES). In vitro

labeling of plasmid DNA was achieved using a modification of the nick-translation method of Chelm and Hallick as used in this laboratory (34). In this procedure, deoxyribonuclease (DNase) enzymatic treatment results in the random phosphodiester bond cleavage of a sample of native DNA. The phosphodiester bonds are reformed by the action of DNA polymerase I. During the repair process, radioactively labeled thymidine 5'-triphosphate, in the reaction mixture, is incorporated in the DNA chain as thymidine 5'-monophosphate. DNA polymerase I has been shown to repair single-strand scissions by incorporating new nucleotide bases at the point of scission and continuing (in the 5' - 3' direction) several bases distal to the point of initiation. This results in radioactively labeled bases being incorporated as part of the double-stranded DNA.

The plasmid DNA was not nicked using DNAse, but the plasmid DNA samples were nicked through manipulation and isoAmyl alcohol extraction following cesium chloride ultracentrifugation. Additional nicking resulted from passage of the plasmid DNA through the gel exclusion column. A reaction mixture was prepared by evaporating to dryness 50 µl of thymidine 5'-triphosphate, tetrasodium salt (methyl ^{3}H; New England Nuclear; 10-20 ci/mM) in a 300 µl Wheaton conical vial in an ice bath under a light stream of nitrogen gas. To this reaction vial was added: 100 µl plasmid DNA (10-20 g/ml), 10 µl Tris buffer (trishydroxymethylaminomethane buffer; 1.4 M; pH 9.4) 5 µl magnesium chloride (0.14 M) 5 µl dithioerythritol (0.01 M), 10 µl dNTP (2 mM each of 2' deoxyadenosine 5'-triphosphate, 2' deocycytidine 5'-triphosphate, 2' deoxyguanisone 5'-triphosphate), 15 µl unlabeled 2'

thymidine 5'-triphosphate (0.6 mM), and 10 µl DNA polymerase I (E. coli B; 200 units/ml; Miles Laboratories). The reaction mixture was incubated in a 30 C water bath for 2 hours. The polymerase was then inactivated by the addition of 10 µl of sodium dodecyl sulfate (20%) and incubation at 65 C for 10 minutes. The total reaction mixture was then layered on a polyacrylamide column (BioRad, P100, 19 x 0.5 cm). The column flow rate was adjusted to 3 ml/hour using a peristaltic pump. The washing buffer consisted of 0.1 x SSC with 0.1% sodium dodecyl sulfate. Column effluent fractions (0.28 ml) were collected using a Golden Pup fraction collector (ISCO Inc.). To each fraction 0.5 ml of 0.1 x SSC buffer was added and mixed. A 5 µl aliquot of each fraction was spotted on Whatman No. 1 paper strips (1 x 0.5 inches) which were dried and placed in vials containing 10 ml Scinti Prep 1 (liquid scintillation counting solution diluted 152 ml/3.8 liters of toluene) and counted in a Beckman liquid scintillation counter (Model LS-255). The fractions following the void volume were then pooled and the DNA concentration was adjusted to 0.2 µg/ml. The labeled DNA solution was sheared by two passages through a French pressure cell at 12,000 psi. Before each use, an aliquot of the labeled DNA was diluted 1:1 with buffer (0.02 M sodium chloride, 10^{-3} M HEPES) and denatured for 5 minutes in a boiling water bath. The labeled DNA was then cooled in an ice bath and used immediately in DNA homology experiments.

Plasmid DNA Homology Procedures. Plasmid DNA's were allowed to reassociate with reference plasmid DNA in a free solution reaction mixture containing 25 µl plasmid DNA (50 to 100 µg/ml), 10 µl labeled plasmid DNA (0.1 g/ml), and 20 µl buffer (1.1 M sodium chloride, 10^{-3}

M HEPES). Renaturation was carried out in rubber capped, 200 µl glass vials submerged for 4 hours in a 60 C water bath.

Following renaturation the vials were removed from the water bath and their contents quantitatively placed into 13 x 100 mm glass tubes containing 1 ml each of S-1 buffer A (0.05 M sodium acetate, 0.5 M sodium chloride, 0.5 mM zinc chloride, 5% glycerol, pH 4.6). To these glass tubes were added 50 µl of sheared denatured salmon sperm DNA (0.5 mg/ml, Calbiochem) and 50 µl of S-1 endonuclease (1:50 dilution of S-1 endonuclease, Calbiochem, diluted in buffer A with 0.3 M sodium chloride).

The reaction mixture was mixed and incubated at 50 C for 1 hour. The duplexed DNA was precipitated by the addition of 1 ml of trichloroacetic acid (TCA) (10%) and 50 µl of sheared native salmon sperm DNA (0.6 mg/ml) which was added as a carrier. The reaction mixture was held on ice for 30 minutes then filtered through 0.45 µm nitrocellulose membrane filters (Schleicher & Schuell, type 85). The membranes were dried under a heat lamp for 45 minutes to 1 hour. The radioactivity on the membranes was counted in Scinti Prep 1 scintillation cocktail in a liquid scintillation counter.

The amount of homology was calculated by subtracting the mean counts per minute of the reaction mixture containing only labeled DNA from the mean counts of the homologous DNA reaction. This was set as 100% homology for easy comparison. All subsequent counts from heterologous competitor DNA reactions were corrected for background by subtracting the counts of the reaction mixture containing only labeled

DNA. This resultant value was then divided by the corrected counts of the homologous reaction and multiplied by 100 to convert it to a percentage value.

Thermal Stability of Plasmid and Bacterial DNA Duplexes. The base composition (mol % G+C) of two plasmid DNA preparations was estimated by comparing the plasmid thermal stability with three bacterial DNA preparations of known mol % G+C. The bacterial DNA preparations used for thermal stability experiments were isolated from Lactobacillus acidophilus (strain 7960; mol % G+C of 35), Enterobacter aerogenes (strain 1627-66; mol % G+C of 45), and Bacteroides thetaiotaomicron (strain 5482; mol % G+C of 42). The DNA preparations were renatured in a 1.23 ml reaction mixture which consisted of 0.36 ml of labeled DNA, 0.6 ml homologous unlabeled DNA (0.6 mg/ml), and 0.36 ml buffer (1.47 M sodium chloride, 10^{-3} M HEPES). These renaturation mixtures were incubated at 25 C below their respective Tm's for 18 hours to achieve a $C_o t$ value greater than 50.

The plasmid DNA isolated from Bacteroides thetaiotaomicron (strain 5482) and B. fragilis type II (strain 4076) was also allowed to renature but to a $C_o t$ value estimated at about 0.2. The plasmid DNA samples were renatured in reaction mixtures containing 0.18 ml labeled plasmid DNA, 0.36 ml of homologous competitor plasmid DNA (10 - 20 μg/ml), and 0.18 ml buffer (1.47 M sodium chloride, 10^{-3} M HEPES). The mixtures were then incubated at 60 C for 5 hours.

Following renaturation, the reaction mixtures were diluted with an equal volume of 50% (v/v) formamide in distilled water and the tubes were placed in a circulating water bath at 40 C. Base line

samples were removed (0.2 ml from bacterial DNA's and 0.1 ml from plasmid DNA's) to 13 x 100 mm glass tubes containing 1 ml S-1 buffer A. The temperature was raised in 5 C increments, DNA samples were allowed to equilibrate to the new temperature for 5 minutes, and then samples were taken. This procedure was repeated for 11 samples from temperatures of 40 C to 90 C. The samples were treated with S-1 endonuclease by adding 50 1 of sheared denatured salmon sperm DNA (0.5 mg/ml) and 50 1 of S-1 endonuclease (diluted 1:50). The samples were then incubated at 50 C for 1 hour. The samples were then precipitated with TCA as previously described. Radioactivity was counted as described also.

Bacteriocin production assay. Bacteriocin production was assayed for all strains that were found to carry plasmid DNA. The methodology used has been previously reported by Brock as cited by Booth (17).

Biochemical and other phenotypic characteristics assayed. All methods for determining phenotypic characteristics such as carbohydrate fermentation, proteolysis, production of indol and catalase, etc. have been previously described in the V.P.I. Anaerobe Laboratory Manual (80).

RESULTS

Bacterial host DNA homology groups. Strains previously identified by the staff of the V.P.I. Anaerobe Laboratory as belonging to species or groups of the saccharolytic Bacteroides were examined for the presence of plasmid DNA. These strains have been placed in one of ten major DNA homology groups or genospecies by Johnson (90). The values of DNA homology shared among the type, neotype, or reference strains of the homology groups are listed in Table 3. All homologous reassociations have been normalized to 100%. These host DNA homology groups have also been referred to as genospecies (89). This term suggests a group of organisms found to be similar based on their genotype specifically rather than their phenotype.

The Bacteroides fragilis genospecies is composed of two sub-groups, type I and type II, which have 70% homology with one another and less than 30% homology with all other genospecies. The majority of isolates of B. fragilis appear to belong to the type I sub-group. Strains of B. fragilis are isolated in greater proportion from clinical specimens (abscess drainage, blood, tissue, etc.) than from fecal specimens (62).

Three additional host DNA homology groups, B. thetaiotaomicron, B. ovatus, and the "3452-A" group (no name has been designated at this time), are interrelated at the 30 to 40% homology level. Each of these groups generally has less than 25% DNA homology with any of the other groups. The B. thetaiotaomicron group is composed of two sub-groups related at 60% homology. Almost all of the strains of B. thetaiotaomicron examined belong to one of the sub-groups (represented

Table 3. Interstrain homology values among the DNA reference organisms[a]

Strain No.	*B. fragilis* 2553	*B. fragilis* 2393	*B. thetaiotaomicron* 5482	*B. thetaiotaomicron* 2302	*B. ovatus* 0038-1	*B. ovatus* C11-2	"3452-A" 3452-A	*B. uniformis* 0061-1	*B. fragilis* subsp. a B5-21	*B. eggerthii* B8-51	*B. vulgatus* 4245	*B. distasonis* 4243	T4-1
2553	100	69	21	36	15	22	10	21	26	18	10	5	15
2393	68	100	19	29	4	19	14	18	29	18	9	17	13
5482	28	28	100	61	40	39	36	22	21	23	12	7	18
2302	21	30	62	100	42	36	47	23	27	21	11	5	16
0038-1	24	15	39	39	100	64	31	17		20	8	4	8
C11-2	20	22	34	40	65	100	43	13					
3452-A	23	20	26	35	37	36	100	11	20	18	0	3	4
0061-1	17	14	22	14	9	13	6	100	38	36	14	6	15
B5-21	28	23	19	27	25	23		30	100	54	19	12	19
B8-51	28	25	23	17	12	13	30	34	49	100	20	8	15
4245	20	14	9	13	8	16	4	19	19	14	100	9	20
4243	18	8	5	4	3	0	3	10	5	3	7	100	20
T4-1	13	12	6	16	7	1		11	18	6	3	23	100

[a]From J. L. Johnson. 1978. Taxonomy of the Bacteroides: I. DNA homologies among *Bacteroides fragilis* and other saccharolytic *Bacteroides* species. Int. J. Syst. Bacteriol. 28:245-256.

by strain 5482). Many isolates, that were identified phenotypically using standard biochemical tests: carbohydrate fermentation, indol production, nitrate reduction, etc., were thought to be B. thetaiotaomicron. However, DNA homology studies have shown that some of these strains actually belong to the species B. uniformis and have less than 20% homology to the B. thetaiotaomicron reference strains. The mol% G+C content of B. uniformis strains is 46% compared with 42% for the B. thetaiotaomicron strains. A high proportion of B. thetaiotaomicron strains have been isolated from clinical specimens while the majority of the B. uniformis strains have been isolated from fecal specimens.

The B. uniformis group has about 30 to 40% DNA homology with strains belonging to B. eggerthii and B. fragilis subspecies "a". The two groups of B. eggerthii and B. fragilis subsp. "a" are interrelated at the 50% homology level, and each has less than 30% homology with the groups other than B. uniformis.

The host DNA homology group of B. vulgatus is composed of strains commonly isolated from human and animal feces. These strains have been reported to constitute 12% of the total bacterial flora of human feces (78, 122). These strains of B. vulgatus appear unrelated to any of the other groups, having less than 20% DNA homology with them.

The two remaining DNA homology groups [B. distasonis and the "T4-1" group (no name has been designated at this time)] are each unrelated to any of the other DNA homology groups or to each other.

Plasmid isolation and detection. Other investigators were able to use the Guerry method with *in vivo* labeled cultures of *Bacteroides* and detect plasmid DNA fractions from cesium chloride gradients (68, 163). However, the Guerry procedure failed to enrich for plasmid DNA over the host DNA from the strains of *Bacteroides* used in this study. It is possible that a major portion of the plasmid DNA of *B. fragilis* was co-precipitated along with the host DNA using the Guerry procedure, while this is not the case with *E. coli*. Many strains of *Bacteroides* have been reported to possess a thick polysaccharide capsule which could contribute to the sedimentation of plasmid DNA (5).

Further attempts were made to isolate plasmid DNA using the Currier and Nester (42) alkaline denaturation-phenol extraction method. With this procedure, it was observed that the growth phase of the organism greatly affected the yield of plasmid DNA.

Plasmid DNA was not found from cultures in the early to mid-logarithmic phase of growth. This poor yield of plasmid DNA could indicate that in young cultures most plasmids are present as a replicating intermediate form and are sensitive to the anionic detergent. The detergent sensitive form of plasmid DNA has been described as a relaxation complex by Clewell and Helinski (36).

The plasmid DNA preparations in this study were further enriched by ultracentrifugation in cesium chloride-ethidium bromide gradients. Ultracentrifugation separates the remaining host and open circular plasmid DNA from the ccc plasmid forms. The greater intercalation of the dye in the non-ccc form of DNA decreases its buoyant density, while the ccc plasmid DNA is less affected. When the plasmid DNA was

treated with ribonuclease A before ultracentrifugation, the contrast between the fluorescent plasmid DNA band and its background was reduced appreciably. In samples that had not been previously treated with ribonuclease, a large amount of RNA (50 to 90% total nucleic acid measured) was observed in the plasmid DNA band after its removal from the gradient and extraction with isoAmyl alcohol. The RNA appears to bind the ethidium dye, as can be seen in precipitates on the bottom of the gradient tube. Perhaps the double-stranded regions of the RNA which are formed by the RNA bending upon itself into a cruciform shape, allow the dye to intercalate as it does with double-stranded DNA. The RNA, binding the fluorescent dye and migrating near the plasmid DNA in the gradient, improves the visibility of the plasmid DNA band. However, the RNA is difficult to separate from the plasmid DNA and interferes in DNA homology experiments (39). The RNA was separated from the plasmid DNA, however, after treating the preparation with ribonuclease A and passing it over an agarose gel exclusion column (exclusion limit 1.5 Mdal). The plasmid DNA (greater than 1.5 Mdal) appeared in early fractions from the column while the migration of the ribonucleotides were retarded. The large ratio of the material in the retarded volume to the material following the void volume demonstrated the magnitude of RNA contamination (Figure 4). RNA, isolated from a non-plasmid containing strain, was purified in neutral sucrose gradients. The larger species (23 s) was placed over the agarose column as a control measure to observe its migration rate in the column. Residual ribonuclease A from previous preparations on the column degraded the 23 s species into oligonucleotides that migrated

in the retarded volume. Less than 1% of total material absorbing light at 260 nm was present in the early fractions. These observations indicate that any RNA contaminant of the plasmid DNA preparations that was digested with ribonuclease before being loaded on the column was degraded to oligonucleotides.

Agarose gel electrophoresis was used to detect the plasmid DNA obtained by the Currier-Nester procedure. Using agarose gel electrophoresis as little as 50 nanograms of plasmid DNA was detected. Plasmid DNA from a strain of bacteria carrying even a single copy of the small 3 Mdal plasmid could be detected.

Agarose electrophoresis is also useful for determining the size of the varous plasmid DNA species (see Figures 1 and 2). This method is less expensive and requires less time per sample to perform than rate zonal centrifugation or contour length measurements using the electron microscope. The sizes of the Bacteroides plasmids were determined using a standard curve constructed from the relative migration rates of E. coli plasmids which were used as standards. The sizes of these plasmids had been determined previously by both sucrose gradients and contour length measurements (115) (see Figure 3).

Distribution and size of plasmid DNA in strains of Bacteroides. A total of 120 strains of Bacteroides were examined for plasmid DNA. Of the 120 strains examined, 52 strains (43%) were found to contain plasmid DNA. Plasmids occurred in each of the ten major homology groups of Bacteroides. One strain (T1-42) which had no DNA homology with any of the ten bacterial DNA homology groups, contained two

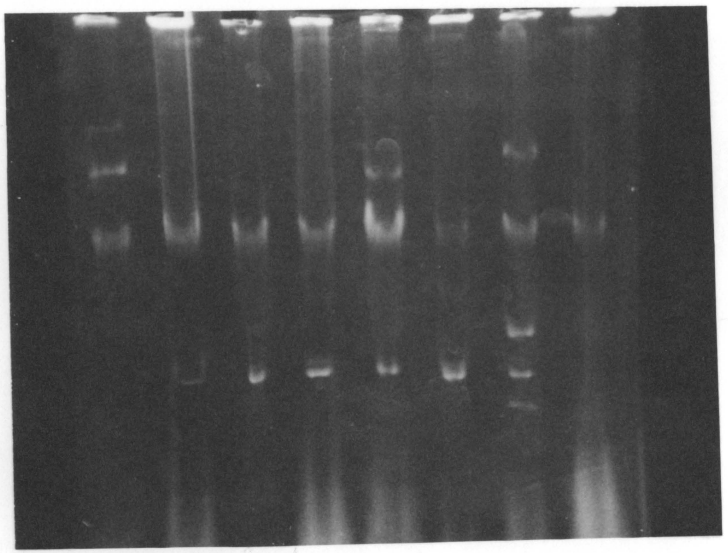

Figure 1. Demonstration of plasmid and host DNA species on agarose gel following electrophoresis (A = R1, 62; Sa, 23; B = J4-2, 2; C = 5008, RNA; D = 2308, 2; E = 3443, 2, 21; F = C54-2, 3; G = C54-1, 2, 3, 7, 30; H = 6266A, none. Number following strain is size in Mdal. The diffuse band in all samples migrating to the middle of the gel is host DNA.)

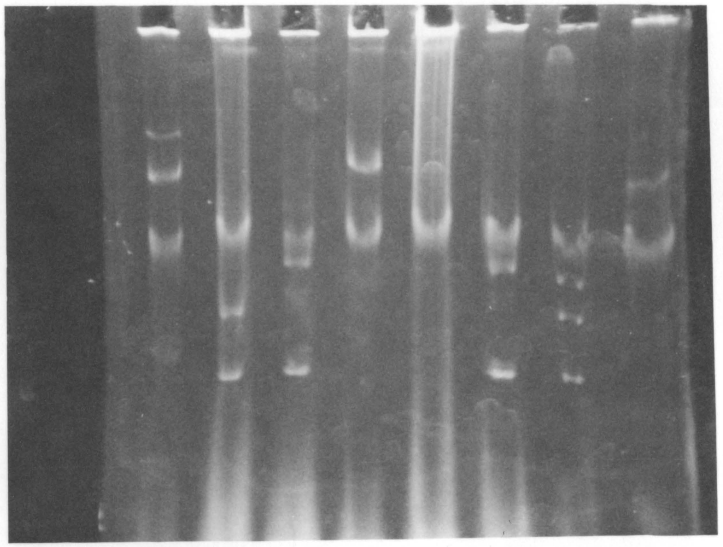

Figure 2. Demonstration of plasmid and host DNA species on agarose following electrophoresis. (A = R1, 62; Sa, 23; B = 2302, 2, 4; C = S1A-52, 3, 7; D = 2553, 23; E = 4243, none; F = X3-31-1A, 3, 7; G = 2882, 2, 4; H = 4245, 21. Number following strain is size in Mdal. The duffuse band in all samples migrating to the middle of the gel is host DNA.)

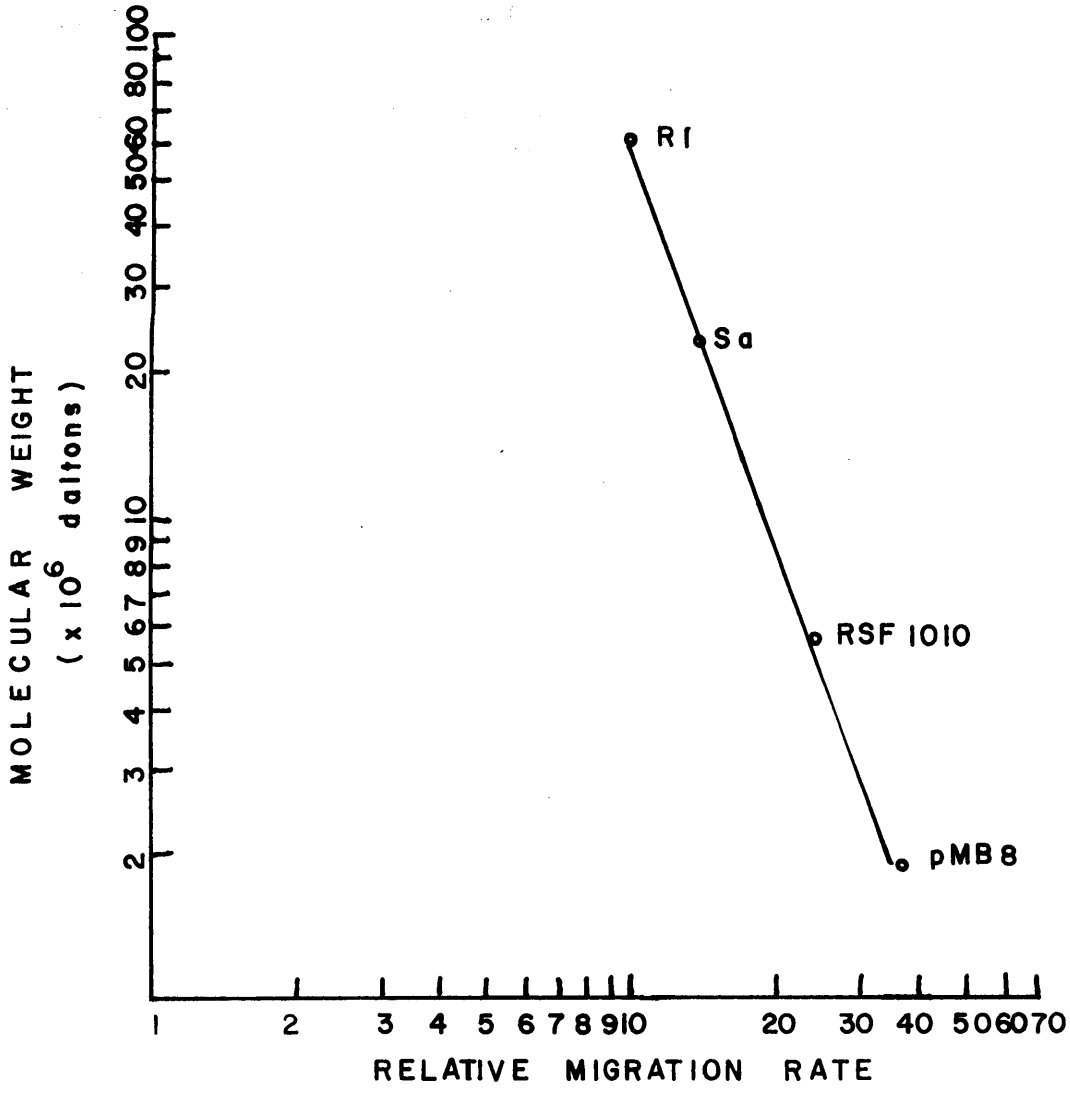

Figure 3. Relative mobility in agarose gel electrophoresis of Escherichia coli plasmids as compared with their molecular weight.

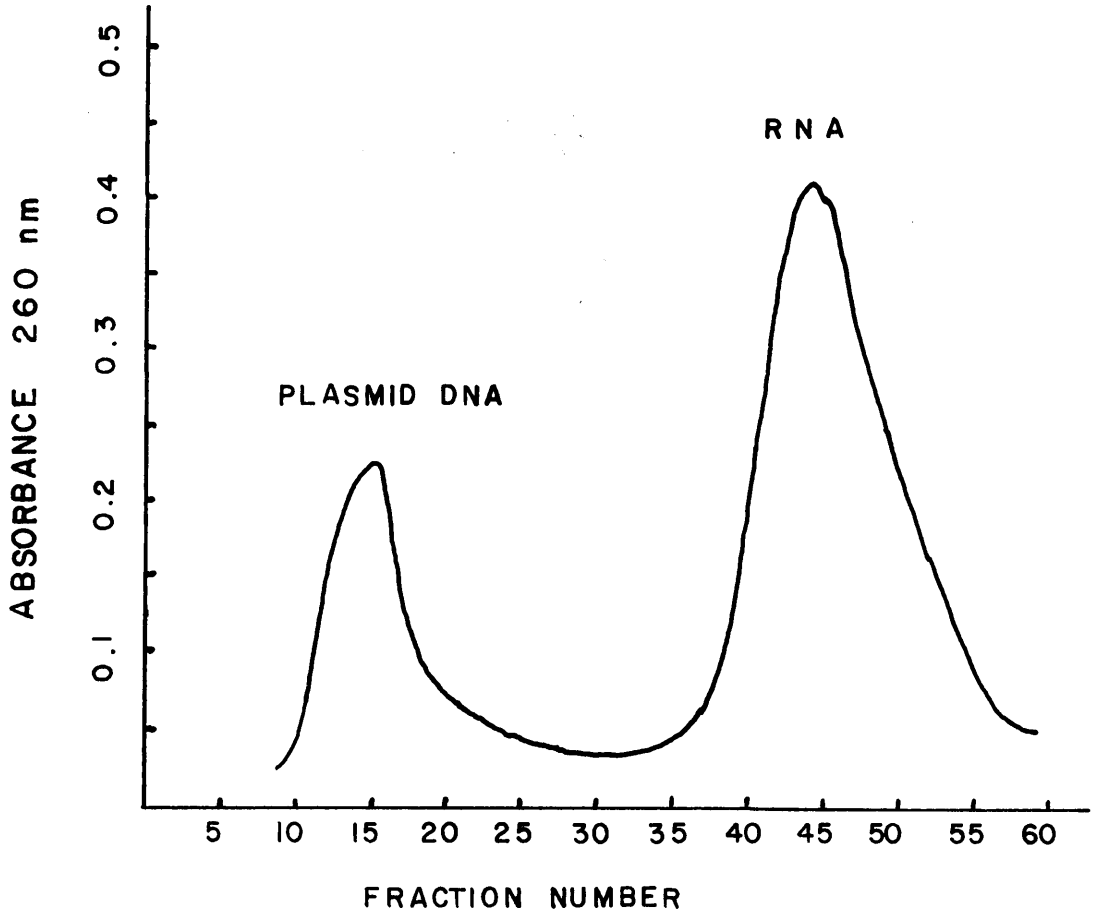

Figure 4. Agarose gel exclusion chromatography of plasmid DNA preparation contaminated with RNA.

plasmids. All of the strains examined and the sizes of plasmids detected are listed in Table 4. As can be seen in Table 4, the occurrence of plasmid DNA varies from strain to strain within each host DNA homology group. Plasmids were found in 8 or 19 strains of the B. fragilis strains examined. Three of five strains of B. thetaiotaomicron carried plasmid DNA, 10 of 26 strains of B. uniformis, 7 of 11 strains of B. distasonis, 8 of 12 strains of the "3452-A" group and 7 of 14 strains of B. vulgatus also contained plasmid DNA. The sample size of each of the species is small and may not reflect a statistically accurate frequency of distribution for unexamined strains of any particular group. However, the total number of strains examined was large enough to suggest that 50% of other similar strains would contain plasmid DNA.

The number of plasmids observed within each plasmid containing strain varied from 1 to 6. The sizes of the plasmids ranged from 2 to 65 megadaltons (Mdal). Many strains contained both a small 2 to 4 Mdal plasmid and a large 20 to 60 Mdal plasmid. One strain, B. vulgatus (6598B), contained 6 plasmid species; 2, 3, 3.6, 4, 7, and 23 Mdal. In two of the major DNA homology groups, one strain in each contained a single plasmid. Strain 2628P, isolated from a hog cecum, belongs to the the "T4-1" homology group, and strain C54-2 is in the B. fragilis subspecies "a" homology group. In addition, strain 4076 was the only strain in B. fragilis subgroup II that contained a plasmid.

Considerations, such as open circular forms, catenated dimer forms and gel size, could affect the estimations of plasmid sizes reported here. Large species of RNA migrated to the same general

Table 4. Distribution and size of plasmid DNA among selected strains of the saccharolytic intestinal Bacteroides

B. fragilis group I

VPI No.	plasmid size (Mdal)
2044	-
2550	-
2553*	23
3390	59
3625	-
4048	27
4082	-
4128	-
4366	-
5001	-
5650	-
6057B	-
6957	3
11666	2,3,5,8
12255	3, 5
12257	5

B. fragilis Group II No.

VPI No.	Plasmid size (mdal)
2393	-
2552	-
4076	22

B. thetaiotaomicron

VPI No.	Plasmid size (Mdal)
0489	-
2302	2,4
3443	2,21
5008	-
5482*	23

B. ovatus

VPI No.	Plasmid size (Mdal)
0038-1*	-
2822	2,4,6
3524	-
4101	-
C11-2	3,23
C40-50A	-
C41-35	2,4
C50-15B	-
J15-5	-
J18-9	-
J22-44	-

"3452-A"

VPI No.	plasmid size (Mdal)
2308	2
3452-A	-
8608	3
C10-2	3,40
C14-3	-
C15-2	-
C27-54	2,4,37
C51-18	2,5
J7-54	2
J15-2	-
T1-12	3
T1-48	3

B. uniformis

VPI No.	Plasmid size (Mdal)
0061-1*	-
0909	-
3537	-
3699A	3,53
5444A	3,53
6387	-
8601	-
A7-10A	-
A13-47	-
B1-15	-
C1-10	2
C7-17	-
C8-30	-
C17-3	-
C20-25	6
C31-12	25
C46-14	2,11,50
C50-6	-
C51-27	2
C54-1	2,3,7,30
J4-2	2
J8-10	-
J15-53	-
OC22A	2,3,37
R5-33	-
T1-1	3

Table 4. Continued

B. eggerthii

VPI No.	plasmid size (Mdal)
B8-51	-
C18-8	-
C19-14	-
C31-22	-
S1A-52	3,7
X3-31-1A	3,7

B. fragilis subsp. a

VPI No.	Plasmid size (Mdal)
B5-21*	-
C1-23	-
C8-19	-
C51-6	-
C52-51A	-
C54-2	3

B. vulgatus

VPI No.	Plasmid size (Mdal)
0959-1	-
2736B	-
4245	21
4506-1	5,18
6186	-
6266A	-
6598B	2,3,3.6,4,7,23
C6-7	30
C7-2	-
C10-6	23
C43-46B	2,23
J17-22	-
J18-14B	2,4,37,65
T1-8	-

B. distasonis

VPI No.	Plasmid size (Mdal)
0052	-
4243*	-
A12-1	5,21
B1-20	2,4
C18-7	2,60
C30-45	3
C50-2	2,3
J15-49A	-
S1-35	-
S6A-50	2,53
T3-25	2,21

"T4-1"

VPI No.	Plasmid size (Mdal)
2628P	62
A19-28B	-
B8-5	-
C16-2	-
C43-5	-
C54-23	-
J20-53	-
R3-5	-
T4-1	-

No designated group

VPI No.	Plasmid size (Mdal)
T1-42	2,5

*Denotes type or neotype strain

area of the agarose gels as the 1.5 to 2.0 Mdal plasmid DNA (see strain 5008 in Figure 1). The similarity of their relative migrations made interpretation difficult, however, there were differences. Plasmid DNA formed a band with a slight upward curve on the ends or appeared completely flat. Large RNA species were distorted either as an oval spot or as a slanted band that was not exactly horizontal or flat. Large RNA species were not visible as discrete bands in cesium chloride-ethidium bromide gradients. They occurred as precipitates and cloudy material near the bottom of the gradient. Treatment of the plasmid DNA preparation with ribonuclease usually decreased the amount of observable RNA both on electrophoresis gels and in cesium chloride-ethidium bromide gradients.

Catenated dimer forms may have been present in the preparations. These forms are duplicates of a small ccc plasmid DNA interlocked with one another and thought to be replicative forms or intermediates of the plasmid DNA (96, 127). A dimer of pMB8, a plasmid that was used as a size standard in this study and isolated from E. coli, was described by Meyers et al. (115). The dimer was measured on agarose electrophoretic gels to be 3.74 Mdal, exactly twice the size of the monomer--1.87 Mdal. In this present study several strains that contained a small (2 to 3 Mdal) plasmid DNA species was observed also to contain a somewhat larger plasmid species (4 to 7 Mdal). Some of these larger plasmids may have been dimers of the 2 to 3 Mdal plasmids. Although these two sizes of plasmid DNA were found together in several of the strains, they also occurred independently. Whether some of the larger plasmids are catenated dimers of smaller forms is not answered by the

data, but this possibility should be considered. One obvious way to show that the larger form is a dimer of the smaller form would be to separate the two DNA species on a sucrose gradient and determine their DNA homology. Similarly, plotting the Cot curves of both the small and large plasmid species should result in a single curve. Electron microscopy could also be used to show catenated forms and heteroduplex analysis would confirm their duplicate nature.

Open circular forms of plasmid DNA up to 10 to 15 Mdal have been reported to migrate 15% slower than the corresponding ccc form (174). Open forms of plasmids greater than 15 Mdal do not appear to migrate into the gel at all (R. Welch, personal communication). Observations during this study confirm this phenomenon. Freshly isolated preparations of plasmid DNA or preparations that have been held at -20 C with a minimum of freezing and thawing, usually do not contain a significant amount of open circular DNA. Plasmid DNA species, that appeared as faint bands migrating 10-15% slower than more intense bands in the same preparation, have been regarded as open circular forms.

The plasmid DNA species that are greater than 20 Mdal are more fragile than the smaller species and convert to the open form more readily. Thus care was taken not to introduce unnecessary shearing forces during plasmid enrichment or measurement. Meyers et al. (115) reported the isolation and measurement of plasmid DNA ranging in size from 0.6 to 95 Mdal using their method. They also reported isolation of plasmids greater than 120 Mdal. These plasmid species, however, do not penetrate the 0.7% vertical agarose gels. Currier and Nester

isolated plasmids ranging in size from 7 to 158 Mdal using their procedure, which was used in this study (41, 42). Theoretically, this study could have detected plasmid DNA ranging in size from 0.6 to 120 Mdal. Large plasmids, however, would not have penetrated the agarose gels during electrophoresis and would not have been observed. The largest plasmid species observed during this study were the 62 to 65 Mdal plasmids of strains 2628 P ("T4-1" group) and J18-48B (B. vulgatus) respectively. In this study, the size of the large plasmids was not measured with the same accuracy as that of Meyers et al. (115) because the gel slabs used here were only one-half the length. Because the relative migration rate of plasmid DNA is logarithmic, greater error of measurement was observed with the larger plasmids. A variation of ± 0.5 mm in migration on these gel slabs would result in a variation from 3.0 to 3.2 Mdal for a 3.1 Mdal plasmid, but a variation from 50 to 60 Mdal for a 55 Mdal plasmid DNA. Meyers et al. (115) reported less than 10% variation in measurement for all sizes, while the variation in this study appears to be 5 to 10% for small plasmid DNA species and 10 to 20% for the larger plasmids. One additional source of error, in measuring plasmid DNA on electrophoresis gel slabs, is the variation provided by overloading the gel with DNA. Overloading is the major source of variation reported by Meyers et al. (115), although they did not believe it to be a significant problem. The true plasmid migration rates in the gels used in this study were determined by varying the sample with concentration and sizes. The relative migration rates of two plasmid species (2.5 and 23 Mdal) remained constant, unless the gel was overloaded with plasmid DNA.

The plasmid would then migrate at a faster rate than the more dilute samples of the same plasmid species. The electrophoretic bond intensities from the samples that migrated at true migration rates were used as a guide to avoid sample overloading.

Reassociation of plasmid DNA. Each plasmid DNA (or mixture of multiple plasmids from the same strain) isolated in this study has been designated with the V.P.I. strain number preceeded by a lower case letter "p" (e.g., p5482). This conforms in part with the standard bacterial plasmid DNA nomenclature as suggested by Novick et al. (126). Each host DNA is designated by the V.P.I. bacterial strain number alone (e.g., 5482). The phrase, "host DNA homology group", has been used to distinguish a classification of strains based on homology of the bacterial host DNA genome. The classification of plasmid species based on DNA homology is referred to as "plasmid DNA homology group".

The reassociation between the radioactively labeled reference plasmid DNA, and unlabeled plasmid DNA was assayed by measuring the increase in S-1 endonuclease resistant DNA. Because naturally occuring plasmid DNA usually has the same mol % G+C as lost DNA (37, 125), plasmid DNA reassociation was carried out at 60 C which is about 25 C below the Tm of the host DNA (84-88 C).

Percent guanine plus cytosine of plasmid DNA as determined by thermalstability studies. Plasmid DNA mol % G+C was determined by comparing the thermal stability of duplexed plasmid DNA (p4076 and p5482) with that of three bacterial DNA preparations of known mol % G+C (Lactobacillus acidophilus 35%, Bacteroides thetaiotaomicron 42%, and Enterobacter aerogenes 45% G+C). The profiles of these thermal

stability data are seen in Figure 5. The plasmid mol % G+C values were obtained from a standard curve correlating mol % G+C of the bacterial reference DNA preparations with their $Tm_{(e)}$. The $Tm_{(e)}$ is the mid-point of a denaturation curve as determined by measuring the separation of the duplexed strands. The reference bacterial DNA preparations had $Tm_{(e)}$ values of 68.3 C for L. acidophilus, 73 C for B. thetaiotaomicron, and 75.5 C for E. aerogenes. These points lay in a straight line when plotted against their respective mol % G+C content. The $Tm_{(e)}$ values of p4076 (isolated from B. fragilis) and p5482 (from B. thetaiotaomicron) were 71 and 73.5 C respectively. From the standard curve in Figure 6, these $Tm_{(e)}$ values correspond to 39 and 43% G+C respectively (±2%). The mid-point of the thermal stability curve (and thus the mol % G+C) for p5482 is almost identical to that of the host DNA (5482). Plasmid DNA from 4076 has a $Tm_{(e)}$ value of 71 C which is two degrees Celcius below the expected value of the 4076 host DNA (73 C). Consequently p4076 contains a % G+C content 3% below the value of the host DNA.

High radioactive background of in vitro labeled reference plasmid DNA preparations. All of the reference plasmid DNA preparations that were labeled in vitro contained fragments that were resistant to S-1 endonuclease digestion. These fragments represented from 23 to 51% of the total radioactive counts of the reference plasmid DNA preparations (see Table 5). These S-1 resistant fragments were initially thought to be palindromic or inverted sequences. Duplexes formed as a result of renaturation of palindromic sequences would appear as cruciform or "hair-pin" like structures with a single-stranded "loop"

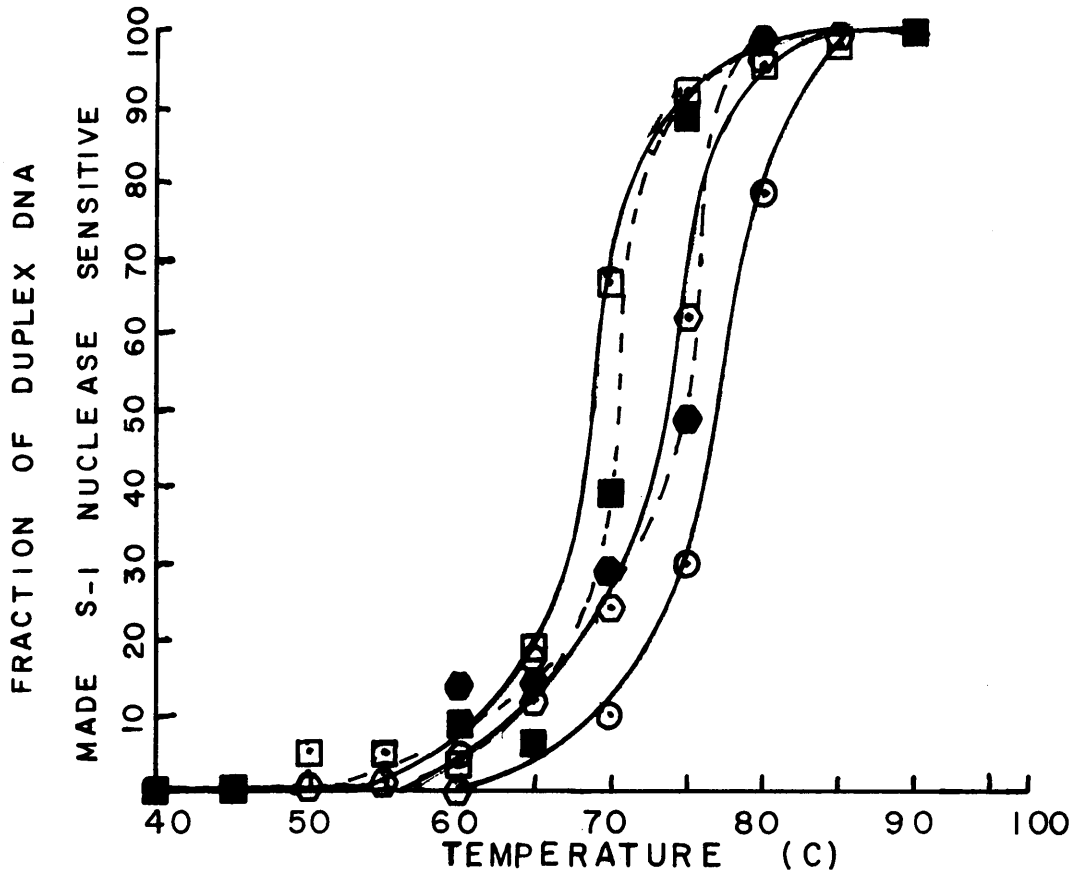

Figure 5. Thermal stability profiles of three bacterial host DNA preparations (from Lactobacillus acidophilus ▭–▭ Bacteroides thetaiotaomicron ⬭–⬭ , and Enterobacter aerogenes ○–○) and two plasmid DNA preparations (from B. fragilis strain 4076 ■–■ and B. thetaiomicron strain 5482 ●–●).

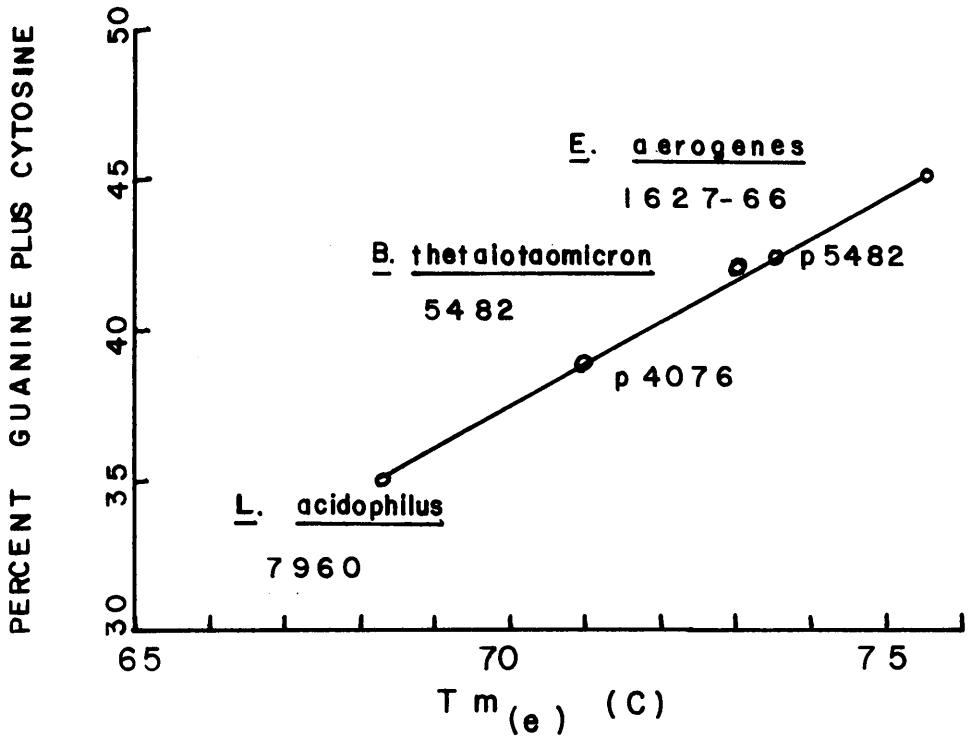

Figure 6. Percent guanine plus cytosine content of bacterial host and plasmid DNA as compared with the mid-point thermal denaturation ($Tm_{(e)}$).

Table 5. Comparison of 10 µl of labeled reference plasmid DNA preparations.

Labeled Plasmid	Total radioactivity incorporated (counts/min.)	Non-specific binding - zero time (label only) (counts/min.)	Homologous reassociation of label with excess unlabeled preparation (counts/min.)
p2553	550	150-250 (36±9%)	400-500 (82±9%)
p4076	650	150-250 (31±8%)	350-450 (62±8%)
p5482	3000	700-1000 (28±5%)	2900-3000 (98±2%)
pT1-1	2400	800-1200 (42±9%)	1600-1800 (71±4%)

region on a "stalk" of duplexed DNA. Such a renaturation or "snap-back" phenomenon could occur quite rapidly. This hypothesis was tested by the following experiment.

The duplexed regions of palindromic fragments would be S-1 resistant. The single-stranded loop region, however, would be S-1 sensitive and would be digested. A thermal denaturation would then result in single-stranded fragments which would be S-1 sensitive. S-1 digestion of palindromic sequences would be observed as a large decrease in radioactive counts after precipitation with TCA. A decrease did not occur as shown in Table 6. Treatment 4 resulted in a decrease of only 200 counts per minute or a decrease of only 15%. This decrease is not sufficient to account for the 33 to 51% of the total counts of the pT1-1 labeled plasmid that was S-1 resistant. The effects of acid hydrolysis can be seen in Treatment 3. The DNA preparation was denatured in the S-1 buffer A (pH 4.6) and was hydrolyzed upon heating at this pH.

To determine whether contaminating RNA could protect the resistant fragments from S-1 digestion plasmid DNA was subjected to alkaline hydrolysis. In this experiment, RNA would be hydrolyzed to nucleotides while the DNA would be left intact. However, there was no decrease in the radioactive counts following precipitation with TCA. The nature of the S-1 resistant DNA fragments still remains unclear.

C$_o$t curve of plasmid DNA. As explained in the introduction, the C$_o$t values indicate the size and complexity of the genome. The C$_o$t curves for T4 bacteriophage (120 Mdal) and E. coli host bacterial DNA (2500 Mdal) have been plotted from data by Britten and Kohne (23)

Table 6. Experiment to detect palindromic sequence in labeled plasmid DNA.

Each reaction mixture contains:

10 μl ^{3}H-pTl-1
20 μl 1.1 M NaCl - 10^{-3} M HEPES
25 μl 0.02 M NaCL - 10^{-3} M HEPES

Treatment		Mean cpm[a]
1	Reaction mixture incubated,[b] TCA precipitated[c]	2729
	Reaction mixture ---------, TCA precipitated	3160
2	Reaction mixture incubated, S-1 treated, TCA precip.	1424
	Reaction mixture ---------, S-1 treated, TCA precip.	1226
3	Reaction mixture incubated, S-1 treated, denatured[d] at pH 4.6, S-1 treated, TCA precip.	447
	Reaction mixture ---------, S-1 treated, denatured at pH 4.6, S-1 treated, TCA precip.	475
4	Reaction mixture incubated, S-1 treated, denatured at pH 7.0, S-1 treated at pH 4.6, TCA precip.	1283
	Reaction mixture S-1 treated, denatured at pH 7.0, S-1 treated at pH 4.6, TCA precip.	899
5	Reaction mixture incubated, S-1 treated, S-1 treated, TCA precip.	1383
	Reaction mixture ---------, S-1 treated, S-1 treated, TCA precip.	1036
6	Reaction mixture incubated, denatured at pH 4.6, S-1 treated, TCA precip.	835
	Reaction mixture ---------, denatured at pH 4.6, S-1 treated, TCA precip.	1111
7	Reaction mixture incubated, denatured at pH 7.0, S-1 treated at pH 4.6, TCA precip.	1990
	Reaction mixture ---------, denatured at pH 7.0, S-1 treated at pH 4.6, TCA precip.	2067

[a] cpm = counts/min.

[b] The reaction mixture was incubated at 60 C for 5 hours ($C_o t = 1 \times 10^{-3}$) before it was used in the protocol above. This incubation step was a control measure to show that any palindromic sequences present would have renatured at room temperature.

[c] TCA precipitation and S-1 treatment are described in Methods.

[d] Denaturation performed at 100C for 5 minutes.

(Figure 7). Data obtained in this present investigation for the 2.5 to 3 Mdal pT1-1 and the 23 Mdal p5482 plasmid DNA preparations are plotted also. Plasmid DNA from 5482 (B. thetaiotaomicron) and T1-1 (B. uniformis) were chosen as being representative of the sizes of plasmid DNA found. The data show $C_o t_{1/2}$ values for pT1-1 and p5482 of 5×10^{-3} and 2.2×10^{-2} (moles-sec/liter) respectively. The $C_o t_{1/2}$ values for T4 bacteriophage and E. coli host DNA are 0.3 and 5 (moles-sec/liter) respectively. Thus the size of the E. coli host DNA is 100 times larger than that of p5482 and 1000 times larger than that of pT1-1 (see Figure 8). The $C_o t_{1/2}$ value of E. coli compares with that of p5482 and pT1-1. These plasmid DNA $C_o t_{1/2}$ values are within the range expected from agarose gel electrophoresis size determinations. Thus the $C_o t_{1/2}$ curves support previous estimations of the sizes of the plasmid DNA.

For plasmid DNA homology experiments it was necessary to define the plasmid DNA concentration and the length of incubation required for complete renaturation. Plasmid DNA of T1-1 was totally renatured at a $C_o t$ value of 0.1 while the p5482 DNA was renatured at a $C_o t$ value of 1.0. The $C_o t$ of 0.1, required for total renaturation of pT1-1, would represent 2.3 g/ml of DNA incubated for 4 hours. Based on these data the concentrations of plasmid DNA were adjusted to achieve complete reassociation in the plasmid DNA homology experiments.

The plasmid DNA $C_o t$ values were obtained by measuring the amount of reassociated DNA using the S-1 assay. The $C_o t$ curves of pT1-1 and p5482 of Figure 7, have been corrected to agree with the hydroxyapatite data of Britten and Kohne (23). While the measurement of duplexed DNA

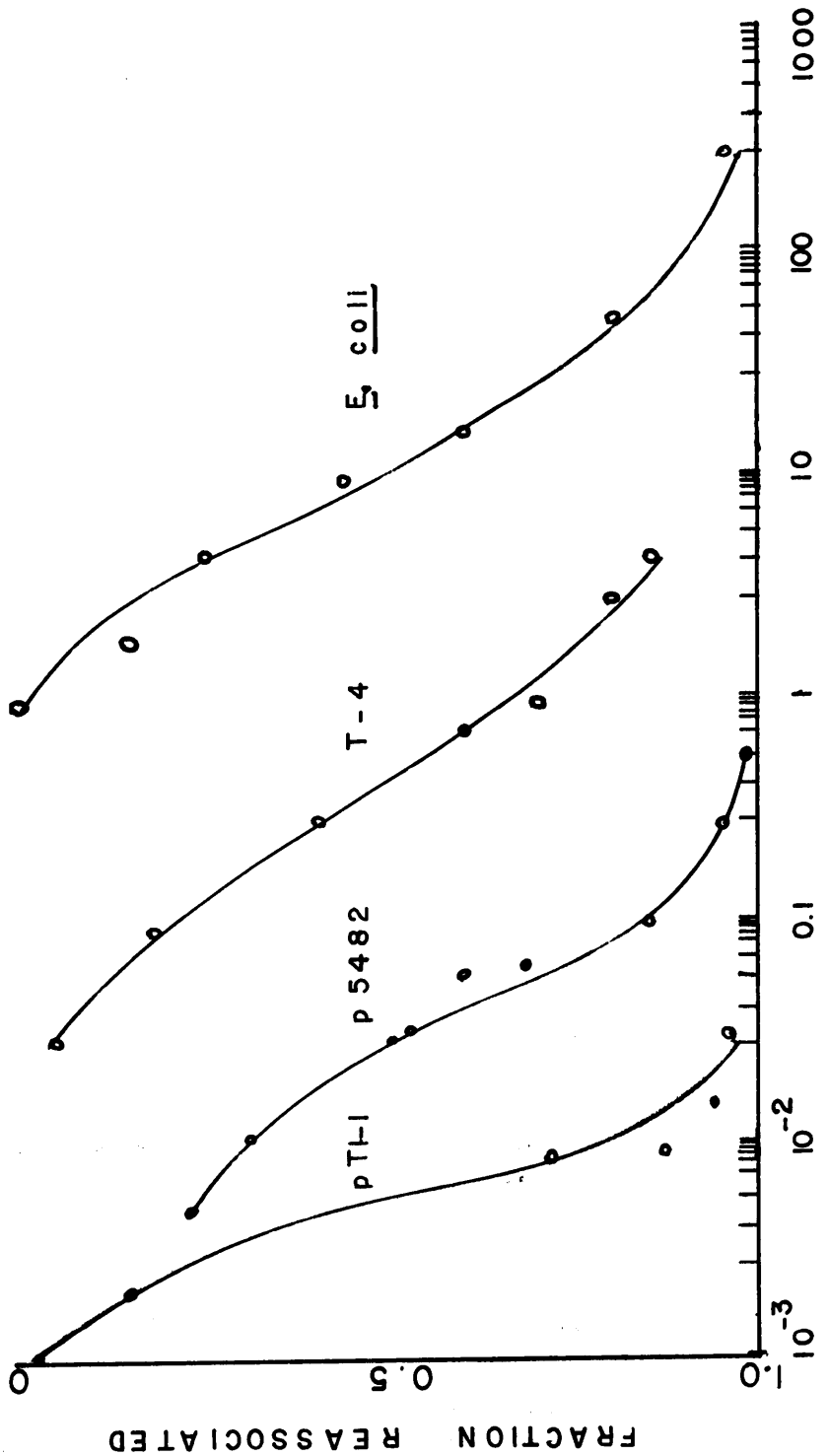

Figure 7. $C_o t$ curve of E. coli host DNA, T4 bacteriophage DNA, and plasmid DNA from B. thetaiotaomicron strain 5482 and B. uniformis T1-1.

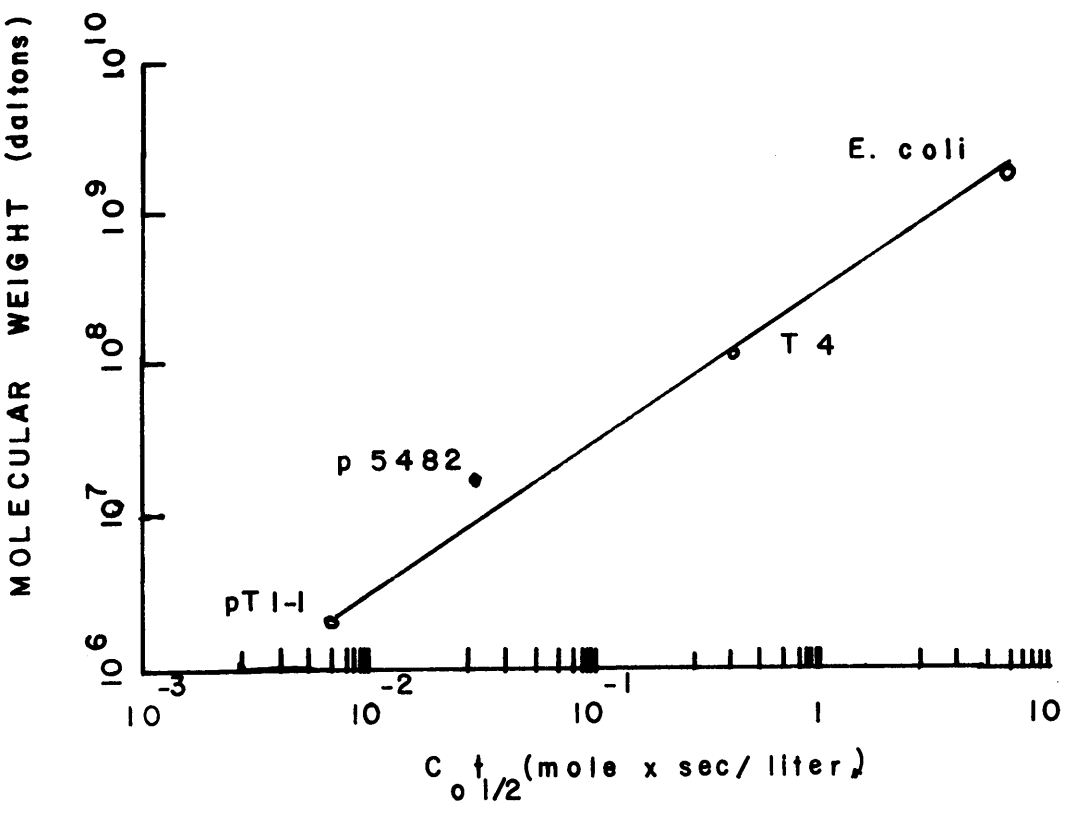

Figure 8. $C_o t$ values at 50% renaturation ($C_o t_{1/2}$) for bacterial, bacteriophage, and plasmid DNA preparations plotted against genome size.

by the S-1 digestion method is comparable to that of the hydroxyapatite method, the reaction rate follows a non-second order form because unduplexed ends of the fragments are being hydrolyzed. This produces a shift of the $C_o t$ curve to the right and indicates a $C_o t_{\frac{1}{2}}$ value that is one half log larger. This shift would suggest a slightly larger DNA genome than actually existed and a slightly longer amount of time required to reach complete reassociation. A correction factor for the S-1 shift has been published as equation 4 by Smith et al. (158). This has been discussed in the Literature Review. For this correction the traditional reassociation equation:

$$\frac{C}{C_o} = \frac{1}{(1 + k_2 C_o t)}$$

is modified to: $$\frac{S}{C_o} = \frac{1}{(1 + K_2 C_o t)}$$

where $\frac{C}{C_o}$ = fraction of single-stranded DNA divided by the total amount of moles of nucleotide, $\frac{S}{C_o}$ = fraction of S-1 sensitive DNA (single-stranded) divided by the total amount of moles of nucleotide; n = 0.45; and k_2 = second order kinetic rate constant.

DNA homology among the reference plasmid DNA preparations. The plasmid DNA preparations chosen as references for *in vitro* labeling belong to three separate and unrelated host DNA homology groups. These plasmids represent the common sizes found among the strains. Three of these plasmids are 22 to 23 Mdal in size (p2553 isolated from *B. fragilis* type I, p4076 from *B. fragilis* type II, and p5482 from *B. thetaiotaomicron*). Plasmid DNA from T1-1 (*B. uniformis*) is about one-tenth the size of the other reference plasmids (2.5 to 3 Mdal). The two *B. fragilis* strains were isolated from clinical specimens and

represent each of the two sub-groups within this genospecies while the strains of B. thetaiotaomicron and B. uniformis were isolated from fecal specimens.

The four reference plasmid DNA preparations have little or no DNA homology (Table 7). The labeled plasmid DNA from T1-1 was 30% homologous with p4076. This result was reproducible and measurably greater than background variation. Because of the difference in size of the two plasmids the reciprocal relationship between labeled p4076 and unlabeled pT1-1 would not be expected and was not seen (see Discussion). Plasmid DNA from the two B. fragilis clinical isolates (p2553 and p4076) were not related nor were the plasmids (p5482 and pT1-1) of the fecal isolates.

Plasmid DNA homology. Plasmid DNA, from each of the 52 plasmid carrying strains of this study, was compared for DNA homology to each of the four reference plasmids. The DNA homology values for these comparisons are listed in Table 8. In this Table the strains are arranged according to the host DNA homology groups. Multiple plasmid species were not separated for homology experiments but used as mixtures from individual bacterial hosts.

All plasmid DNA preparations that had greater than 20% homology were tested again. Additionally, these unlabeled plasmid DNA preparations were allowed to renature with their respective label DNA at a concentration one-tenth of that used in previous experiments. This was to determine if complete renaturation had been reached in the previous experiments. If the concentration of the unlabeled DNA preparations had been high enough to reach complete renaturation,

Table 7. DNA homology among the reference plasmids

	Size (Mdal)	p2553	p4076	p5482	pT1-1
p2553	23	100	0	4	0
p4076	22	0	100	2	31
p5482	23	0	0	100	12
pT1-1	3	0	0	3	100

Table 8. Plasmid DNA Homology

VPI Strain	Plasmid size (Mdal)	Reference Plasmid DNA p2553	p4076	p5482	pT1-1
B. fragilis I					
p2553	23	100	0	0,6	0
p3390	59	0	0	0	15
p4048	27	101,109[a]	0	0	7
p6957	3	0	0	2	100,91
p11666	2,3,5,8	0	0	13	82,99
p12255	3,5	0	0	0	90,84
p12257	5	0	0	2	11
B. fragilis II					
p4076	22	0	100	1,5	29,32
B. thetaiotaomicron					
p2302	2,4	1	0	6	107,99
p3443	2,21	6	0	126,125	101,87
p5482	23	0	0	100	12
B. ovatus					
p2822	2,4,6	0	0	6	103,86
pC11-2	3,23	115,132	0,0	9	87,109
pC41-35	2,4	0	6	4	26
"3452-A"					
p2308	2	3	0	6	115,117
p8608	3	0	0	2	97,114
pC10-2	3,40	59,78*	2	8	84,69
pC27-54	2,4,37	12	0	37,33*	125,100
pC51-18	2,5	0	0	9	98,93
pJ7-54	2	4	0,0	0	17,28*
pT1-12	3	14,0	7,0	1,8	100,87
pT1-48	3	12,1	0,0	4,9	(44,43)*,104
B. uniformis					
p3699A	3,53	6	0	3	58,65
p5444A	3,53	0	0	3	47,53
pC1-10	3	12	4	3	0
pC20-25	6	12	0	9	0
pC31-12	25	0	0	29,28*	32
pC46-14	2,11,50	0	0	8	96,86
pC51-27	2	0	0,0	2	98,95
pC54-1	2,3,7,30	77,75	0,0	2	82,69
pJ4-2	2	0	0,0	0	81,74
pOC22A	2,3,37	73,74	0,0	0	105,100
pT1-1	3	0	0	2,4	100

Table 8. Continued

VPI No.	Plasmid size (Mdal)	Reference Plasmid DNA p2553	p4076	p5482	pT1-1
B. eggerthii					
pS1A-52	3,7	9	0,7	0	102,106
pX3-31-1A	3,7	0	4	4	99,84
B. fragilis subsp. a					
pC54-2	3	0	0,0	7	70,61
B. vulgatus					
p4245	21	3	0	116,103	39
p4506-1	5,18	2	0	69,75*	98,98
p6598B	2,3,3.6,4,7,23	11	0	73,83*	88,76
pC6-7	30	70,71*	0	0	25,31*
pC10-6	23	49,64*	0	1	20
pC43-46B	2,23	87,97	0	10	15
pJ18-14B	2,4,37,65	68,77	0,0	1	81,70
B. distasonis					
pA12-1	5,21	1	0	12	36,51*
pB1-20	2,4	11	6	3	27,34*
pC18-7	2,60	11	0	7	0
pC30-45	2,3	1	0	27,27*	0
pC50-2	3	0	4	0	0
pS6A-50	2,53	4	0,0	0	1
pT3-25	2,21	12	7	86,96	10,19*
"T4-1"					
p2628B	62	0	0	5	12
No designated group					
pT1-42	2,5	9	2,0	0	67,78

*Denotes estimates that are probably too low because unlabeled DNA concentrations may have been too low to allow complete reassociation.

[a]Plasmid preparations, that were found to have homology (at least 20% or greater) with the reference plasmid, were repeated.

[b]Preparations that contain multiple plasmids may not have equal amounts of each species. However any plasmid species may be present in a sufficient concentration to indicate that the preparation is 100% homologous with a reference plasmid. A preparation could also contain more than one plasmid in sufficient concentrations to indicate 100% homology to two reference plasmids (e.g., p3443).

a ten-fold decrease in concentration would reduce the amount of renaturation only moderately because of the exponential nature of the $C_o t$ curve. However, if renaturation had not been complete because of the low concentration of unlabeled DNA, its $C_o t$ value would decrease sharply by a ten-fold reduction in concentration. For values that decreased in percent of homology indicating that complete renaturation may not have been reached (and thus may have homologies that are higher than reported), an asterisk (*) is placed beside the percent homology value.

The plasmid DNA homology groups did not correlate with any of the bacterial host DNA homology groups. The reference plasmid DNA isolated from T1-1 appears to be the most widely distributed plasmid DNA in the study. Of the 52 plasmid carrying strains examined, 34 strains were found to contain plasmid DNA having either moderate (30-60%) or high (61-100%) DNA homology with the T1-1 plasmid DNA. These plasmids occurred in nine of the 10 major host DNA homology groups of this study. Plasmid DNA isolated from strain 5482 has moderate to high DNA homology with plasmid DNA of 10 strains from 6 of the 10 major host DNA homology groups. Plasmid DNA isolated from strain 2553 has moderate to high DNA homology with plasmid DNA of 10 strains from among 5 of the 10 major host DNA homology groups. None of the 10 plasmid DNA preparations with homology to p5482 had any homology with p2553. The converse was also true: none of the 9 plasmid DNA preparations with homology to p2553 had any homology to p5482. The DNA in these 21 strains appears to be mutually exclusive whether this is by evolutionary design or chance is unknown. The reference plasmid DNA

of 4076 was not found to have homology with any of the unlabeled plasmid DNA preparations. However, labeled pT1-1 reference DNA does have 30% homology with p4076.

Single plasmid DNA carrying strains. A natural assumption might be that plasmid DNA of a given size has homology with a plasmid of the same size in a multiple plasmid preparation. However, the data of this study are not sufficient to support that tenant. In single plasmid preparations, the data do support this assumption. In Table 9, single plasmid DNA preparations that have homology with any of the reference plasmids are listed. There are several interesting exceptions to this "size rule". Plasmid DNA from B. fragilis strain 4076, and B. vulgatus strains 4245, C6-7 and C10-6 have high homology with a large reference plasmid and plasmid DNA from B. uniformis strain C31-12 has moderate homology to p5482. In addition to the homology with a large reference plasmid, these plasmids also have 20-30% homology with pT1-1 DNA. The small plasmids, with only one exception: C54-2 (of B. fragilis subsp. "a"), have moderate to high homology only with the small reference plasmid, pT1-1. Small plasmids however would not be expected to show homology to larger plasmids because of the difference in size of the reference plasmid and the unlabeled DNA. A labeled plasmid ten times larger than the unlabeled plasmid DNA would not carry the same concentration of homologous sequences as would be the converse case of a small labeled plasmid reassociating with a large unlabeled plasmid.

Multiple plasmid carrying strains. Several strains with multiple plasmid species appear to have plasmid DNA that has homology with more

Table 9. Single Plasmid DNA Species with Homology to Reference Plasmid DNA

Strain	Size Mdal	Genosp.	p2553	p4076	p5482	pT1-1
2553	23	*B. fragilis*	100	0	0, 6	0
4048	27	*B. fragilis*	101, 109[a]	0	0	7
6957	3	*B. fragilis*	0	0	2	100, 91
4076	22	*B. fragilis*	0	100	1, 5	29, 32
2308	2	"3452-A"	3	0	6	115, 117
8608	3	"3452-A"	0	0	2	97, 114
J7-54	2	"3452-A"	4	0	0	17, 45
T1-12	3	"3452-A"	14, 0	0	1, 8	100, 87
T1-48	3	"3452-A"	12, 1	0	4, 9	104
C31-12	25	*B. uniformis*	0	0	29, 28	20
C52-27	2	*B. uniformis*	0	0	2	98, 77
J4-2	2	*B. uniformis*	0	0	0	81, 74
T1-1	3	*B. uniformis*	0	0	2, 4	100
5482	23	*B. thetaiotaomicron*	3	0	100	12
4245	21	*B. vulgatus*	3	0	116, 103	20
C6-7	30	*B. vulgatus*	70, 71	0	0	25, 31
C10-6	23	*B. vulgatus*	49, 64	0	1	20
C54-2	3	*B. fragilis* subsp. a	0	0	27, 47	70, 61

[a]Multiple values are repeat determinations.

than one reference plasmid DNA. It already has been shown that the reference plasmids do not have any homology with each other except a small amount of homology between pT1-1 and p4076. Strains listed in Table 10 show plasmids with multiple homologies to the reference plasmids. These 9 strains all contain both small and large plasmids. These plasmid DNA preparations have moderate to high homology with a (either p2553 or p5482) large reference plasmid and the small reference plasmid (pT1-1). This may indicate homology between plasmids of similar sizes. No plasmid preparations had homology with both large reference plasmids.

Correlation of plasmid DNA homology groups with source of isolation. The data were analyzed to detect any pattern or correlation of plasmid DNA homology grouping with respect to source of isolation. All of the 52 plasmid carrying strains were isolated from human feces except the following which were isolated from human infections: B. fragilis strains 2553, 4048, 6957, 12255, 12257, 4076; "3452-A" strain 8608; B. ovatus strain 2822; B. vulgatus strain 4506-1, and 6598B, isolated from hog cecum; "T4-1" strain 2628P. The source of isolation was unknown for B. fragilis strain 11666; and B. uniformis strain 5444A.

Many infections of man are thought to be caused by members of the genus Bacteroides from the indogenous intestinal flora. Whether a distinction can be made between human fecal isolates and human infection isolates is unclear. There was no correlation between the source of isolation of the strains and either plasmid size or plasmid homology group. All of the T strains (T1-1, B. uniformis; T1-12 and T1-48,

Table 10. Homology Values Among Multiple Plasmid Strains

Strain	Size (Mdal)	Genosp.	p2553	p4076	p5482	pT1-1
C10-2	3, 40	"3452-A"	59, 78[a]	2	8	84, 69
C27-54	2, 4, 37	"3452-A"	12	0	37, 33	125, 89
C54-1	2,3,7,30	B. unif.	77, 121	0	2	98, 77
OC22A	2,3,37	B. unif.	73, 106	0	0	105, 66
C11-2	3, 23	B. ovatus	115, 132	0	9	87, 109
3443	2, 21	B. theta.	6	0	126, 125	101, 87
4506-1	5, 18	B. vulgatus	2	0	69, 75	98, 98
6598B	7, 23 2,3,3.6,4	B. vulgatus	2	0	73, 83	88, 76
J18-14B	2,4,37,65	B. vulgatus	68, 112	0	1	81, 70

[a]Multiple values are repeat determinations.

"3452-A" group; T1-42, no designated group; T1-8, B. vulgatus; T3-25, B. distasonis; T4-1 "T4-1" group), were isolated from fecal samples from one man during a fecal flora study. As seen from Table 4, five of these strains carried plasmids (T1-1, T1-12, T1-42, T1-48, and T3-25) while the other two did not (T1-8 and T4-1). Strain 2628P (from the "T4-1" host DNA homology group) isolated from the hog cecum is the only animal isolate carrying plasmid DNA in this study. The plasmid of 2628P does not have any homology with any of the four reference plasmids. Plasmid DNA was not found in the one other animal isolate examined (0052, B. distasonis).

Plasmid DNA homology grouping with respect to bacteriocin production. Booth, Johnson, and Wilkins (17) studied bacteriocin production in a collection of strains of Bacteroides. Three strains; T1-1 (B. uniformis), T1-12, and T1-48 (both of the "3452-A" group) produced strong bacteriocin activity with an identical indicator host range, yet they belong to two unrelated bacterial DNA homology groups. The type strain of B. uniformis (0061) and the reference strain of 3452-A did not produce bacteriocins. These five strains were examined by Macrina (personal communication) for plasmid DNA. Each of the bacteriocin producing strains was found to carry a 3 Mdal plasmid species, while the two strains that did not produce bacteriocin also did not possess any plasmid DNA. This apparent correlation of bacteriocin production with a 3 Mdal plasmid species was investigated further by assaying for bacteriocin production among all 52 plasmid carrying strains of this survey. The production of bacteriocin activity of an identical indicator host range was observed for 4076

(B. fragilis), Tl-1 (B. uniformis), Tl-12 ("3452-A"), Tl-42 (no designated host homology group), and Tl-48 ("3452-A"). There appears to be no correlation of the presence of a 3 Mdal plasmid with bacteriocin production (see Table 11). There were many strains of bacteria carrying a 3 Mdal plasmid with high homology to the Tl-1 plasmid that did not produce a bacteriocin (e.g., 2303 (B. thetaiotaomicron); 2308, 8608 (both of the "3452-A" group); C51-27, J4-7 (both of B. uniformis; and C54-2 (of B. fragilis subsp. "a").

Lytic areas were not replated on indicator lawns to differentiate bacteriophage activity from bacteriocin activity. Strain 4076 was observed to lyse spontaneously in several cultures during the logarithmic phase of growth, suggesting bacteriophage lysis. This was not confirmed, however, by serial plaque transfers.

The results of this survey for bacteriocin production agree with the results reported by Booth et al. except that strain 3390 of B. fragilis did not produce bacteriocin active against the indicator strain 3392 in this study. The reason for this variation is not known. No bacteriocin production by strains previously found to be negative was observed. Bacteriocin production did not correlate with any particular plasmid DNA homology group.

Correlation of plasmid DNA homology group with other phenotypic characteristics. A computer analysis of 81 phenotypic traits was made, comparing each with the four plasmid DNA homology groups. No correlation was observed. Those traits included the ability to ferment any of 24 carbohydrates, hydrolyze esculin and starch and digest gelatin, milk and meat. Other phenotypic characters include such

Table 11. Bacteriocin production in strains of *Bacteroides*[a]

Strains examined for bacteriocin production	Indicator strains											
	2393	2647-J2	3392	4147	4736	4932	6001-A	6057-B	A10-36B	C8-19	C19-3	C48-32
2302[b]	-	-	-	-	-	-	-	-	-	-	-	-
2308[b]	-	-	-	-	-	-	-	-	-	-	-	-
2553	-	+	-	+	wk	-	wk	-	-	-	-	-
3390	-	-	-	-	-	-	-	+	+	-	-	-
3443[c]	-	-	-	-	-	-	-	-	-	-	-	-
4048[d]	-	-	-	-	-	-	-	-	-	-	-	-
4076	+	+	+	+	+	+	+	+	+	+	+	+
4245	-	+	-	-	-	+	-	-	-	-	-	-
4506-1	-	-	-	-	-	+	-	-	-	-	-	-
6598B	-	+	-	-	-	+	-	-	-	-	-	-
6957	-	-	+	-	-	-	-	wk	+	-	-	+
C6-7	-	+	-	-	-	+	-	-	-	-	-	-
C18-7	+	-	-	-	-	-	-	-	-	-	-	-

Table 11. Continued.

Strains examined for bacteriocin production	Indicator strains											
	2393	2647-J2	3392	4147	4736	4932	6001-A	6057-B	A10-36B	C8-19	C19-3	C48-32
T1-1	+	+	+	+	+	+	+	+	+	+	+	+
T1-2	+	+	+	+	+	+	+	+	+	+	+	+
T1-42	+	+	+	+	+	+	+	+	+	+	+	+
T1-48	+	+	+	+	+	+	+	+	+	+	+	+
T3-25	-	-	-	-	-	-	-	-	-	-	-	-

[a]All strains found to produce bacteriocin are listed.

[b]Carries a plasmid species with high homology to pT1-1.

[c]Carries two plasmid species with high homology to p5482 and pT1-1.

[d]Carries a plasmid species with high homology to p2553.

traits as production of metabolic end products from a fermentable carbohydrate, catalase, indol, lecithin, and lipase. Antibiotic susceptibilities were compared on a few strains of each of the plasmid DNA homology groups.

DISCUSSION

The advent of improved methods for anaerobic bacteriology and bacterial taxonomy has brought the genus Bacteroides and in particular the species B. fragilis out of obscurity and into the forefront of current microbiology. Many workers consider B. fragilis a significant constituent (at least quantitatively) of the large intestine and of many anaerobic infections of man. With the increased awareness of the significance of B. fragilis and related species and with the ease of culturing these organisms, it is appropriate that they be the focus of multiple disciplinary research in microbiology. B. fragilis and related species have been studied from taxonomic, physiological, clinical, and molecular biological points of view. Considering B. fragilis from a genetic point of view, one is presented with an enigmatic challenge. Little is known of the genetics of B. fragilis at the present time because only recently has genetic transfer within these related species been observed.

There have been several reports on the presence of plasmid DNA in strains of the Bacteroides (45, 68, 104, 148). Several workers have transferred bacterial genetic information between strains of Bacteroides and Escherichia coli (27, 104, 113). Recent reports by Welch and Macrina (173) and Tally et al. (160) describe the transfer of multiple antibiotic resistance between strains of B. fragilis. With an active transfer system, the genetics of the Bacteroides can be studied in more detail. While an active transfer system obviously shows exchange of genetic information among strains of Bacteroides, one also can infer such a transfer from the distribution of plasmid DNA species

among the strains (3). Demonstration of plasmid DNA distribution through studies on the DNA homology of the plasmid species, was one of the objectives of this research effort.

Plasmid DNA distribution and size in strains of the Bacteroides. The detection of plasmid DNA in 43% of all the strains investigated in this study indicates that the presence of plasmids in this group of organisms is a common property. The sizes of plasmids observed varied from 2 to 65 Mdal and thus represented sizes commonly seen in other groups of bacteria. The size estimations of the plasmids from this study were supported also by data from $C_o t$ curves (see Figure 8). No plasmids greater than 65 Mdal were observed, although the methods employed in this study have been used previously to isolate and detect plasmids of 1.8 through 120 Mdal (42, 114).

Characteristics of plasmid DNA preparations. The mole percent guanine plus cytosime (mol % G+C) was determined for the reference plasmids p4076 and p5482. Clowes (37) and Novick (124) suggested that most plasmid DNA species have the same G+C content as the host DNA. The data of this study support this assumption. The mol % G+C of p5482 is 43%, while that of host DNA (strain 5482) is 42% which is with an experimental error of ± 2%. The mol % G+C of p4076 is 39%, which is 3% below the value of the host DNA of strain 4076.

The four labeled plasmid DNA preparations all contained a fraction of DNA fragments that were resistant to S-1 endonuclease digestion. The resistant fraction varied from 20 to 50% of the total DNA (measured as radioactivity). The nature of this S-1 resistant is unknown but the

present data suggest that the resistance was not caused by RNA contamination. Some preparations of bacterial host DNA labeled either in vivo or in vitro in this laboratory have demonstrated a fraction of S-1 resistant fragments comprising up to 20% of the total DNA. The resistance might be attributed to covalent crosslinking between complimentary strands, co-precipitation of DNA with a material that protects it from the S-1 enzyme or to non-specific binding between single-stranded DNA fragments. Resistant fractions also occurred in hydroxyapatite column experiments in that a fraction of the denatured DNA adsorbed to hydroxyapatite under conditions where only single-stranded DNA should adsorb. Some workers have removed this fraction from labeled DNA by passing the preparation over a column of hydroxyapatite (HA) before using it in homology experiments (39). The S-1 resistant and HA adsorbed fragments probably represent the same DNA fraction. The significance of the S-1 resistant fraction is unclear. It is doubtful that any specific region of the plasmid DNA is selectively present in the S-1 resistant fraction (from $C_o t$ data of this study and Dr. S. Falkow, personal communication).

Plasmid DNA homology groups. The plasmid DNA from 52 strains of Bacteroides was isolated and used in homology experiments. The reference plasmids were p2553 - 23 Mdal, p4076 - 22 Mdal, p5482 - 23 Mdal, and pT1-1 - 3 Mdal. These plasmids represent the most common sizes seen in this study. Plasmid DNA from 2553 and 4076 (B. fragilis) and 5482 (B. thetaiotaomicron) were chosen as reference plasmids because strains from both of these genospecies are often isolated from clinical specimens. Strain 2553 and 5482 are the neotype strains of

the two genospecies respectively. These four plasmids exist as single plasmid species in their hosts, therefore it was not necessary to separate them further from other plasmid species.

The plasmid DNA from T1-1 was chosen in part because it was a small plasmid and a preliminary examination indicated that it might have been involved in bacteriocin production. The plasmid DNA from 4076 was chosen also to determine whether the plasmid DNA species from the two DNA homology groups of the B. fragilis genospecies were related. The choice of these four reference plasmids was fortunate in that 44 of the 52 plasmid DNA preparations had some homology with them. The reference plasmids themselves, however, had no homology with each other, except for the small amount (30%) between pT1-1 and p4076 (Table 7). This degree of homology would indicate that 1 Mdal of the T1-1 plasmid has homology with p4076. The plasmid DNA homology groups did not correlate with host homology lines (Table 8). Nine of the ten genospecies contained plasmid DNA which had moderate (30 to 60%) to high (61 to 100%) homology with the small T1-1 plasmid species. According to Anderson et al. (3), such distribution of homology indicates that genetic transfer has occurred among strains of these nine genospecies. This plasmid or part of it was distributed among 25% of the 120 Bacteroides strains.

The other reference plasmid DNA species were not as widely distributed. Plasmid DNA from 5482 has homology with plasmid DNA from 5 of the 10 host DNA homology groups. In addition to having homology with fewer strains than pT1-1, p5482 also appears to have quantitatively less homology with the plasmids that belong to its

homology groups. Plasmid DNA from three of the nine strains of the p5482 group have only moderate homology with p5482. However, this moderate homology might be a reflection of the low concentration of plasmid DNA in these preparations because data from 5 of the 9 plasmid preparations that have homology with p5482 lay in the low renaturation (upper) region of the $C_o t$ curve. Most of the plasmid preparations that have homology with the labeled pT1-1 plasmid reached complete renaturation and lay in the asymptotic (lower) region. Where the $C_o t$ data indicated incomplete renaturation, an asterisk (*) was placed beside the homology values in Table 8 to suggest that a higher value might have been obtained if renaturation were allowed to continue for a longer time.

Nine plasmid DNA preparations had 50-100% homology with plasmid DNA from 2553. Ten strains of this group were distributed among 5 of the 10 host DNA homology groups. The majority of these strains had renatured completely as indicated by the $C_o t$ curve data. Six strains of the p2553 homology group that have 75% homology with p2553 contain plasmid species of 30 to 40 Mdal in size. Seventy-five percent of the p2553 DNA is integrated into the 30 to 40 Mdal plasmids of these 6 strains. These strains each contained multiple plasmid species, therefore homologous plasmid DNA on the other plasmid species in addition to the 30 to 40 Mdal plasmids might have been detected.

Plasmid DNA from 4076 does not have homology with any strains of this study except the labeled reference plasmid from T1-1. The pT1-1 plasmid has approximately 30% homology with p4076. The reciprocal relationship, however, between labeled p4076 and unlabeled pT1-1 is

negligible. The lack of reciprocity can be explained on the basis of plasmid sizes. Because p4076 binds 30% of the labeled pT1-1, this indicates that one-third of pT1-1 has enough homology with p4076 for the bases to pair in register. However, one-third of a 3 Mdal plasmid is 1 Mdal, but 1 Mdal is only 5% of the 22 Mdal p4076. Five percent of the labeled p4076 is all that could bind pT1-1. This estimated homology value of 5% is within the limits of error observed in this study. Because p4076 would be expected to have 5% homology with pT1-1, it would also be expected to have a similar amount of homology with all other small plasmids that have a high degree of homology with pT1-1. Thus, p4076 would be expected to have 5% homology with labeled preparations of pT1-12, pT1-48, etc. which have 90 to 100% homology with pT1-1.

When grown in chopped meat carbohydrate broth or other complex medium with glucose, cells of strain 4076 appeared to lyse spontaneously during the early to mid-logarithmic phase of growth. Spontaneous lysis is indicative of a bacteriophage infection. Additionally, areas of lysis in confluent lawns of sensitive indicator strains were observed (as will be discussed in this section) during examination for bacteriocin production. These observations indicate that p4076 may be a replicative form or intermediate of a bacteriophage rather than a plasmid DNA. Serial transfer of lytic plaques and the demonstration of bacteriophage particles under electron microscopy would confirm that p4076 is a bacteriophage.

The 5% homology that p4076 has with pT1-1 demonstrates that large plasmids can have regions of homology with smaller plasmids but one

might assume that plasmids of similar sizes would be more likely to be related. To determine the validity of this assumption, homology data for the single plasmid preparations have been separated from the multiple plasmid species and listed in Table 9. The single plasmid DNA preparations have high DNA homology with only one reference plasmid, usually the reference plasmid of a similar size. These single plasmid species also have moderate or low homology with a second reference plasmid, either smaller or larger than the unlabeled DNA plasmid.

An unlabeled plasmid that has homology with two reference plasmids could inciate hybrid of composite plasmids arising through natural recombination events. One possible example of this phenomenon can be seen in the 30 Mdal plasmid from B. vulgatus strain C6-7. This plasmid DNA appears to have incorporated 75% of the p2553 DNA and 1 Mdal from pT1-1. These sequences only account for 18 Mdal of the 30 Mdal plasmid. Other pieces of DNA may have arisen from host or other plasmid DNA sources. Because of the wide distribution of some plasmid replicative genes and insertion sequences, it is tempting to speculate that the sequences that appear distributed throughout many of these plasmids homologous to pT1-1 may also have similar functions. For example, at least 1 Mdal of pT1-1 appears to be shared among the plasmids homologous to pT1-1 and plasmids of large plasmid homology groups such as: p4076 (B. fragilis); C6-7, and C10-6 (both from B. vulgatus).

Some insertion sequences and antibiotic resistance genes are highly conserved and can be found in plasmid DNA isolated from many difference bacterial hosts (46, 54, 64). This conservation of genes has

led some researchers to question the use of restriction endonuclease profiles as a means of comparing plasmid DNA similarity (31). One "modular" piece of plasmid DNA found in many plasmids is the ampicillin transposon (TnA or TnAp) (73). A transposon is a piece of DNA bearing specific genes which migrate intact in and out of a genome (bacterial host as well as plasmid). This modular character is attributed to inverted repeating sequences which flank the specific genes on the transposon and are homologous with regions of thegenome. TnAp has a minimum of 19 different sites of homology and integration with the RSF 1010 plasmid (5.5 Mdal) (143). As homologous sequences align, a pairing and recombinational event occurs with the insertion of the transposon. While ampicillin resistance does not appear to be plasmid-mediated in these strains of the Bacteroides, the integration of TnAp into the host chromosome can not be precluded. The TnAp example, however, does suggest a possible explanation for such well distributed homologous sequences among the plasmids from the present study.

Correlation of plasmid DNA homology groups and bacteriocin production. There was no correlation of bacteriocin production by any of the bacterial strains with plasmid DNA homology groups. Booth et al. (17) have reported that bacteriocins were produced by a minority of the bacterial strains they examined. In this present study 12 out of 52 strains produced bacteriocins (Table 11).

All strains with designations that start with a "T" were isolated from fecal specimens from one individual. Seven "T" strains (representing six host DNA homology groups) were examined for plasmid DNA. Five of these strains carried plasmid DNA. Four of these

plasmid carrying strains had a small plasmid with high homology to pT1-1 and produced a bacteriocin of the same host range. The other plasmid carrying strains did not produce bacteriocin and the plasmids it carried were not homologous to pT1-1. The strains with no plasmid DNA were not assayed for bacteriocin production. However other strains carrying plasmid DNA species with 80-100% homology to pT1-1 did not produce a bacteriocin that was active upon the 12 indicator strains.

Two strains of B. fragilis (2553 and 4048) each have a 23 Mdal plasmid. These two plasmids have high homology with one another. However, only strain 2553 produced a bacteriocin.

Strain 4076 (B. fragilis) produced lytic areas on all of the indicator strains. Whether this lysis resulted from the production of bacteriocin or bacteriophage infection is unknown.

Even though no correlation was found between the production of bacteriocin and plasmid DNA homology groups, plasmid genes mediating bacteriocin production could be integrated into the host chromosome. Strains that did not produce bacteriocin but contained plasmids with high homology to pT1-1, could carry defective bacteriocin genes on those plasmids. Further investigation into the cross testing of bacteriocin producing strains and non-producing strains should be conducted. In the report by Booth et al. (17) strains: T1-1, T1-12, and T1-48 were immune or insensitive to bacteriocin produced by other strains. However, strain: 3443 (B. thetaiotaomicron) was also resistant to bacteriocin and carried a plasmid which had high homology to pT1-1, even though it did not produce bacteriocin itself (Booth,

unpublished data). This resistance could of course arise through defective or lack of bacteriocin receptors or the impermeability of the cell wall to bacteriocin. Porter et al. (134) reported the presence of a defective colicin plasmid in Shigella dysenteriae which provides the cell with immunity to the colicin but does not provide for the production of an active colicin. In this present study, three of the indicator strains were included for the examination of plasmid DNA, however these strains (2393, 6057B, C8-9) did not carry any plasmid DNA. Perhaps the sensitivity of these three strains to bacteriocin results from the absence of plasmid DNA bearing immunity genes.

Correlation of plasmid DNA homology groups and other phenotypic characteristics. There was no correlation between any of the plasmid DNA homology groups and any of 81 phenotypic characters examined. A computer analysis failed to detect any plasmid DNA homology group of strains which was positive for a given phenotypic trait that was not also present in the control group of strains. The control group consisted of both strains which did not possess plasmid DNA and strains which did carry plasmid DNA that was unrelated to the plasmids of the compared group.

The antibiotic susceptibilities of strains of each plasmid DNA homology group were also compared with negative control groups of strains. The data on antibiotic susceptibilities were limited to a smaller number of strains than most of the other phenotypic traits. However, if antibiotic resistance was mediated by any of the plasmids this relationship should have been detected. Many strains of

Bacteroides are resistant to penicillin and some to tetracycline. None of the plasmid DNA homology groups correlated with resistance to penicillin, tetracycline, chloramphenicol, clindamycin, or erythromycin. As with the production of bacteriocin, strains containing plasmid DNA that had integrated into the host genome would demonstrate the same phenotypic characters as those strains whose plasmids existed as autonomous molecules in the cell.

Redundant DNA sequences and genes present on plasmid as well as host DNA could make the correlations of phenotypic characters and plasmid DNA homology groups difficult. For example, a cell could conceivably contain genes encoding for the fermentation of rhamnose both on the host DNA and on plasmid DNA. Another similar strain without the plasmid DNA but with rhamnose genes on the host DNA would still appear positive for the trait of rhamnose fermentation. When the two strains were compared rhamnose fermentation could not be ascribed to the plasmid. In such cases the determination of any homology between plasmids and host DNA would help to indicate the extent of common sequences, the presence of shared phenotypic traits, and possibly the significance of cryptic plasmids.

For the present the plasmid DNA species of this study are cryptic. Their genes encode for proteins, whose activity was not assayed. Such traits as cell surface antigens, extracellular enzyme production and bacteriophage susceptibility and many others could be examined for possible plasmid DNA mediation. Other plasmids, however, found in similar strains of Bacteroides have been found to carry genes for clindamycin and tetracycline resistance (160, 173). Stiffler et

al. (158) isolated plasmid DNA from three strains of Bacteroides but were unable to correlate their presence with resistance to any of the commonly used antibiotics. Further work needs to be done in correlating phenotypic traits with the presence of plasmid DNA. This study has shown from plasmid DNA homology experiments that many strains contain similar plasmids. Using conjugation to transfer cryptic plasmids into receptive hosts, one might be able to discern the functions of these plasmids. Eliminating or "curing" the cell of its plasmid DNA with acriflavin dyes might also help elucidate the phenotypic role mediated by the plasmid DNA of these intestinal saccharolytic Bacteroides.

LITERATURE CITED

1. Akiba, T., K. Koyama, Y. Ishiki, S. Kimura, and T. Fukushima. 1960. On the mechanism of the development of multiple drug resistant clones of Shigella. Jap. J. Microbiol. 4:219-227. [quoted by Watanabe (169)]

2. Akiba, T., K. Koyama, S. Kimura, and T. Fukushima. 1961. Studies on the mechanism of transfer of drug-resistance in bacteria. III. Experiments on the transfer of drug-resistance in vivo. Med. Biol. (Tokyo) 59:185-188. [quoted by Watanabe (169)]

3. Anderson, E. S., G. O. Humphreys, and G. A. Willshaw. 1975. The molecular relatedness of R factors in Enterobacter of human and animal origin. J. Gen. Microbiol. 91:376-382.

4. Ando, T. 1966. A nuclease specific for bent denatured DNA isolated from a product of Aspergillus oryzae. Biochim. et. Biophys. Acta. 114:158-168.

5. Babb, J. L. and C. S. Cummins. 1978. Encapsulation of Bacteroides species. Inf. and Imm. 19:1088-1091.

6. Barnes, W. 1977. Plasmid detection and sizing in single colony lysates. Science 195:393-394.

7. Bauer, W. and J. Vinograd. 1971. Circular DNA in Basic Principles of Nucleic Acid Chemistry. Tso (ed.), Vol. II. Academic Press pp. 265-303.

8. Bazaral, M. and D. Helinski. 1968. Circular DNA forms of colicinogenic factors E1, E2, and E3 from Escherichia coli. J. Mol. Biol. 36:185-194.

9. Beerens, H. and G. Baron. 1965. Mise en evidence de bacteriocines elaborees par les bacteries anaerobies a gram negatif appartenant au genre Eggerthella. Ann. Inst. Pasteur Paris 108:225-256.

10. Beerens, H., G. Baron, and M. M. Tahon. 1966. Mise en evidence du substances inhibitrices elaborees pour les bacteries anaerobies non sporules. Ann. Inst. Pasteur Lille 17:1-20.

11. Beerens, H., Y. Schaffner, J. Guillaume, and M. M. Castel. 1963. Les bacilles anaerobies non sporules a gram negatif favorises par la bile. Leur appartenance au genre Eggerthella (nov. gen.) Ann. Inst. Pasteur Lille 5-48.

12. Beerens, H., P. Wattre, T. Shinjo, and C. Ramond. 1971. De classification serologique de 131 souches de Bacteroides du groupe fragilis (Eggerthella). Ann. Inst. Pasteur 121:187-198.

13. Bernardii, G. 1969. Chromatography of nucleic acids on hydroxyapatite. I. Chromatography of native DNA. Biochim. et. Biophys. Acta. 174:423-434.

14. Bernardii, G. 1969. Chromatography of nucleic acids on hydroxypatite. II. Chromatography of denatured DNA. Biochim. et. Biophys. Acta. 174:435-448.

15. Bernardii, G. 1969. Chromatography of nucleic acids on hydroxyapatite. III. Chromatography of RNA and polyribonucleotides. Biochim. et. Biophys. Acta. 174:449-457.

16. Bolton, E. and B. McCarthy. 1962. A general method for the isolation of RNA complementary to DNA. Proc. Natl. Acad. Sci. U.S.A. 48:1350-1397.

17. Booth, J., J. L. Johnson, and T. D. Wilkins. 1977. Bacteriocin production by strains of Bacteroides isolated from human feces and the role of these strains in the bacterial ecology of the colon. Antimicrob. Agents & Chemotherapy 11:718-724.

18. Bourgois, K. and B. Beamer. 1976. In vitro spheroplast and L-forms induction within the pathogenic nocardiae. J. Bacteriol. 127:584-594.

19. Branche, W. D., Jr., V. M. Young, H. G. Robinst, and E. M. Massey. 1964. Effect of colicine production of Escherichia coli in the normal human intestine. Soc. Exp. Biol. Med. 114:148-201.

20. Brenner, D. J., G. R. Fanning, A. Rohe, and K. E. Johnson. 1969. A batch procedure for thermal elution of DNA from hydroxyapatite. Anal. Biochem. 28:447-459.

21. Brenner, D. J., G. R. Fanning, K. E. Johnson, R. V. Citarella, and S. Falkow. 1969. Polynucleotide sequence relationship among members of the Enterobacteriaceae. J. Bacteriol. 98:637-650.

22. Brenner, D. J., M. A. Martin, B. H. Hoyer. 1967. Deoxyribonucleic acid homologies among some bacteria. J. Bacteriol. 94:486-487.

23. Britten, R. J. and D. E. Kohne. 1968. Repeated sequences in DNA. Science 161:529-540.

24. Britten, R. J., M. Povich, and J. Smith. 1969. A new method for DNA purification. Cornegie Inst. Washington Yearbook 68:400-402.

25. Brubacker, R. R. 1972. The genus Yersinia: biochemistry and genetics of virulence. Current topics Microbiol. and Immunol. 57:111-158.

26. Burt, S. J. and D. R. Woods. 1976. R factor transfer to obligate anaerobes from Escherichia coli. J. Gen. Micro. 93:405-409.

27. Burt, S. J. and D. Woods. 1977. Transfection of the Anaerobe Bacteroides thetaiotaomicron. J. Gen. Microbiol. 103:181-187.

28. Campbell, A. 1969. Episomes. Transfer Agents. In H. Halvoson, H. Roman, E. Bell (eds.) Modern Perspectives in Biology. Harper and Row Publishers, New York, N.Y.

29. Castellani, A. and J. A. Chalmers. 1919. Manual of Tropical Medicine. 3rd Ed., p. 959-960. William Wood and Co., N.Y.

30. Cato, E. P. and J. L. Johnson. 1976. Reinstatement of species rank for Bacteroides fragilis, B. ovatus, B. distasonis, B. thetaiotaomicron, and B. vulgatus: Designation of neotype strains for Bacteroides fragilis (Veillon and Zuber) Castellani and Chalmers and Bacteroides thetaiotaomicron (Distaso) Castellani and Chalmers. Int. J. Syst. Bacteriol. 26:230-237.

31. Causey, S. and L. Brown. 1978. Transconjugant analysis: Limitations on the use of sequence-specific endonuclease for plasmid identification. J. Bacteriol. 135:1070-1079.

32. Chakrabarty, A., G. Chou, and I. Gunsalus. 1973. Genetic regulation and extrachromosomal nature of octane degradative pathway in Pseudomonas. Proc. Natl. Acad. Sci. U.S.A. 70:1137-1140.

33. Chassey, B., E. Gibson, and A. Giuffrida. 1976. Evidence for extrachromosomal elements in Lactobacillus. J. Bacteriol. 127:1576-1578.

34. Chelm, B. K. and R. B. Hallick. 1976. Changes in the expression of the chloroplast genome of Euglena gracilis during chloroplast development. Biochemistry 15:543-549.

35. Chilton, M. D., A. L. Montoya, D. J. Merlo, M. H. Drummond, R. Nutter, M. P. Gordon, and E. W. Nester. 1978. Restriction endonuclease mapping of a plasmid that confers ocogenicity upon Agrobacterium timefaciens strain B6-80G. Plasmid 1:254-269.

36. Clewell, D. and D. Helsinki. 1969. Supercoiled circular DNA-protein complex in Escherichia coli: Purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. 62:1159-1166.

37. Clowes, R. 1972. Molecular structure of bacterial plasmids. Bacteriol. Rev. 36:361-405.

38. Cooke, E. M., I. G. T. Hettiaratchy, and A. C. Buck. 1972. Fate of ingested Escherichia coli in normal persons. J. Med. Microbiol. 5:361-369.

39. Crossa, J. H., D. J. Brenner, and S. Falkow. 1973. Use of a single-strand specific nuclease for analysis of bacterial and plasmid DNA homo- and heteroduplexes. J. Bacteriol. 115:904-911.

40. Cummins, C. S. and J. L. Johnson. 1971. Taxonomy of the Clostridia: Wall composition and DNA homologies in Clostridium butyricum and other butyric acid-producing Clostridia. J. Gen. Microbiol. 67:33-46.

41. Currier, T. C. and E. W. Nester. 1976. Evidence for diverse types of large plasmids in tumor-inducing strains of Agrobacterium. J. Bacteriol. 126:157-165.

42. Currier, T. C. and E. W. Nester. 1976. Isolation of high molecular weight plasmid DNA. Analytical Biochemistry 76:431-441.

43. Curtiss, R., III. 1976. Genetic manipulation of microorganisms: Potential benefits and biohazards. Annu. Rev. Microbiol. 30: 507-533.

44. Dajani, A. S. and Z. Taube. 1974. Plasmid-mediated production of staphylococcein in bacteriophage type 71 Staphylococcus aureus. Antimicrob. Agents and Chemother. 5:594-598.

45. Damle, S. P. and S. A. Syed. 1975. Demonstration of plasmid DNA in Bacteroides species of oral origin. J. Dent. Res. 54:104-special issue A.

46. DeGraff, J., C. P. Elwell, and S. Falkow. 1976. Molecular nature of two beta-lactamase-specifying plasmids isolated from Haemophilus influenzae. J. Bacteriol. 126:439-446.

47. Del Bene, V. E., M. Rodgers, and W. E. Fanon. 1976. Attempted transfer of antibiotic resistance between Bacteroides and Escherichia coli. J. Gen. Microbiol. 92:384-390.

48. DeLey, J., H. Cattoir, and A. Reynaerts. 1970. The quantitative measurement of DNA hybridization from renaturation rates. Eur. J. Biochem. 12:133-142.

49. Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. and Biophys. Res. Commun. 23:641-646.

50. Dessev, G. and K. Grancharov. 1973. Precipitation of RNA, DNA, and nucleoprotein particles from very dilute solutions. Anal. Biochem. 53:269-271.

51. Doty, P., J. Marmur, J. Ergner, and C. Schildkraut. 1960. Strand separation and specific recombination in deoxyribonucleic acids: Physical chemical studies. Proc. Natl. Acad. Sci. U.S.A. 46:461-476.

52. Eggerth, A. H. and B. H. Gagnon. 1933. The bacteroides of human feces. J. Bacteriol. 25:389-413.

53. Elwell, L. P., J. DeGraaf, D. Seibert, and S. Falkow. 1975. Plasmid-linked ampicillin resistance in Haemophilus influenzae type b. Inf. and Imm. 12:404-410.

54. Elwell, L. P., J. R.Saunders, M. H. Richmond, and S. Falkow. 1977. Relationships among some R plasmids found in Haemophilus influenzae. J. Bacteriol. 131:356-362.

55. Falkow, S. (ed.) 1975. Infectious multiple drug resistance. Pion. Ltd. London.

56. Falkow, S. and R. V. Citarella. 1965. Molecular homology of F-merogenote DNA. J. Mol. Biol. 12:138-151.

57. Finegold, S. 1969. Intestinal bacteria the role they play in normal physiology, pathogenic physiology and infection. California Med. 110:455-459.

58. Finegold, S., V. C. Sutter, J. D. Boyle, and K. Shimado. 1970. The normal flora of ileostomy and transverse colostomy effluents. J. Inf. Dis. 122:376-381.

59. Fredericq, P. and M. Betz-Barean. 1953. Transfert genetique de la propriete colocinogene chez Escherichia coli. Compt. Rend. Soc. Biol. 147:1110-1112.

60. Freifelder, D. R. and D. Freifelder. 1968. Studies on Escherichia coli sex factors. I. Specific labeling of F'lac DNA. J. Mol. Biol. 32:15-23.

61. Gillespie, D. and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. 12:829-842.

62. Gorbach, S. L. and J. G. Bartlett. 1974. Medical progress: Anaerobic infections. N. Eng. J. Med. 290:1177-1184; 1237-1245; 1289-1294.

63. Gratia, A. 1932. Antagoniome microbien et bacteriophagie. Ann. Inst. Pasteur Paris 48:413-437.

64. Grindley, N. D., G. O. Humphreys, and E. S. Anderson. 1973. Molecular studies of R factor compatability groups. J. Bacteriol. 115:387-398.

65. Grinsted, J., J. Saunders, R. Ingram, R. Sykes, and M. Richmond. Properties of an R factor which originated in Pseudomonas aeruginosa 1822. J. Bacteriol. 110:529-537.

66. Guerry, P., D. J. LeBlane, and S. Falkow. 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116:1064-1066.

67. Guerry, P. and S. Falkow. 1971. Polynucleotide sequence relationships among some bacterial plasmids. J. Bacteriol. 107:372-374.

68. Guiney, D. G. and C. E. Davis. 1975. Isolation of plasmid deoxyribonucleic acid from two strains of Bacteroides. J. Bacteriol. 124:503-510.

69. Guiney, D. G. and C. D. Davis. 1978. Identification of a conjugative R plasmid in Bacteroides ochraceus capable of transfer to Escherichia coli. Nature (London) 274:181-182.

70. Haenel, H. and J. Bendig. 1975. Intestinal flora in health and disease. Progress in food and nutrition. Science 1:21-64.

71. Hardy, K. G. 1975. Coliciongeny and related phenomena. Bacteriol. Rev. 39:464-515.

72. Hayes, W. 19753. Observations on a transmissible agent determining sexual differentiation in Bact. coli. J. Gen. Microbiol. 8:72-82.

73. Heffron, F., C. Rubens, and S. Falkow. 1977. Transposition of a plasmid deoxyribonucleic acid sequence that mediates ampicillin resistance: Identity of laboratory-constructed plasmids and clinical isolates. J. Bacteriol. 129:530-533.

74. Hellinski, D. R. 1973. Plasmid determined resistance to antibiotics: molecular properties of R factors. Annu. Rev. Microbiol. 27:437-470.

75. Hentges, D. J. 1970. Enteric pathogen-normal flora interaction. Am. J. Clin. Nutr. 23:1451-1456.

76. Hickson, F. T., T. R. Roth, and D. R. Helinski. 1967. Circular DNA forms of a bacterial sex factor. Proc. Natl. Acad. Sci. U.S.A. 58:1731-1738.

77. Hill, G. B., S. Osterhort, and P. C. Pratt. 1974. Five abscess production by non-spore-forming anaerobic bacteria in a mouse model. Inf. Immun. 9:959-603.

78. Holdeman, L. V., I. J. Good and W. E. C. Moore. 1976. Human fecal flora: Variation in bacterial composition within individuals and a possible effect of emotional stress. Appl. and Environ. Microbiol. 31:359-375.

79. Holdeman, L. V. and W. E. C. Moore. 1974. In R. E. Buchanan and N. E. Gibbons (eds.). Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkins Co., Baltimore pp. 387-389.

80. Holdeman, L. V., W. E. C. Moore, and E. P. Cato (ed.). 1977. Anaerobe Laboratory Manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, VA.

81. Hoyer, B. H., B. J. McCarthy, and E. T. Bolton. 1963. Complementary RNA in nucleus and cytoplasm of mouse liver cells. Science 140:1408-1412.

82. Hoyer, B. H., B. J. McCarthy, and E. T. Bolton. 1964. A molecular approach in the systematics of higher organisms. Science 144:959-967.

83. Hutton, J. R. 1977. Renaturation kinetics and thermal stability of DNA in aqueous solutions of formamide and urea. Nucleic Acid Res. 4:3537-3555.

84. Inselbry, J. 1971. R factor deoxyribonucleic acid in chromosomeless progeny of Escherichia coli. J. Bacteriol. 105:620-628.

85. Ionesco, H. and D. H. Bouenchaud. 1973. Production de bacteriocine lise a la presence d'un plasmide chez Clostridium perfringens type A. Compt. Rend. Acad. Sci. 276:2855-2857.

86. Jacob, A. E., G. Douglas, and S. Hobbs. 1975. Self-transmissible plasmids determining the hemolysin and bacteriocin of Streptococcus faecalis var. zymogene. J. Bacteriol. 121:863-872.

87. Jacob, F. and E. L. Wollmon. 1956. Recombinaison genetique et mutants de fertilite chez E. coli K-12. Comptes. Rend. 242: 303-310.

88. Janez, H., S. Pouwel, and J. Schiphorst. 1966. Separation of double-stranded DNA (replicative form) of bacteriophage phi X 124: a simplified method. Biochem. Biophys. Acta. 122:626-627.

89. Johnson, J. L. 1973. Use of nucleic acid homologies in the taxonomy of anaerobic bacteria. Int. J. Syst. Bacteriol. 23:308-315.

90. Johnson, J. L. 1978. Taxonomy of Bacteroides. I. DNA homologies among Bacteroides fragilis and other saccharolytic Bacteroides species. Int. J. Syst. Bacteriol. 28:245-256.

91. Johnson, J. L. and E. J. Ordal. 1968. Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity. J. Bacteriol. 95:893-900.

92. Jones, G. W. and J. M. Rutter. 1972. Role of the K88 antigen in pathogenesis of neonatal diarrhea caused by Escherichia coli in piglets. Infect. Immun. 6:918-927.

93. Kagiwada, S., S. Kato, M. R. Rokugo, J. Hoshino, and K. Nishiyama. 1960. Studies on the mechanism of development of resistant Shigella in intestinal tracts with special reference to the possibility of converting sensitive Shigella to resistance by multiple drug-resistant E. coli in intestinal tracts. Nippon Iji Shimpo 1886:5-9. [quoted by Watanabe (169)].

94. Kline, B. and D. Helinski. F sex factor of Escherichia coli size and purification in the form of a strand specific relaxation complex of supercoiled deoxyribonucleic acid and proteins. Biochemistry 10:4974-4980.

95. Kline, B., J. Miller, D. Cuss, M. Wlodarczyk, J. Manis, and M. Otter. 1976. Nonintegrated plasmid-chromosome complexes in Escherichia coli. J. Bacteriol. 127:881-889.

96. Kupersztoch, Y. and D. R. Helinski. 1973. A catenated DNA molecule as an intermediate in the replication of the resistance transfer factor R6K in E. coli. Biochem. Biophys. Res. Commun. 54:1451-1459.

97. Lacey, R. 1975. Antibiotic resistance plasmids of Staphylococcus aureus and their clinical importance. Bacteriol. Rev. 39:1-32.

98. Lederberg, J. 1952. Cel genetics and hereditory symbiosis. Physiol. Rev. 32:403-430.

99. Lederberg, J., L. L. Cavolli, and E. M. Lederberg. 1952. Sex compatability in E. coli. Genetics 37:720-730.

100. Lehninger, A. L. (ed.). 1977. In Biochemistry, 2nd ed. Worth Pub. Co. N.Y., N.Y. p. 336.

101. Levy, S. B. 1971. Physical and functional characteristics of R factor deoxyribonucleic acid segregated in Escherichia coli minicells. J. Bacteriol. 108:300-308.

102. McCarthy, B. J. and E. T. Bolton. 1963. An approach to the measurement of genetic relatedness among organisms. Proc. Natl. Acad. Sci. U.S.A. 50:156-164.

103. McCarthy, B. and R. B. Church. 1970. The specificity of molecular hybridization reactions. Annu. Rev. Bioch. 39:131-150.

104. McConaughy, B., C. Laird, and B. McCarthy. 1969. Nucleic acid reassociation in formamide. Biochemistry 8:3289-3294.

105. Mancini, C. and R. J. Behme. 1977. Transfer of multiple antibiotic resistance from Bacteroides fragilis to Escherichia coli. J. Infect. Dis. 136:597-600.

106. Mandel, M., L. Igambi, J. Bergendahl, M. L. Dodson, and E. Scheltgen. 1970. Correlation of melting temperature and cesium chloride buoyant density of bacterial deoxyribonucleic acids. J. Bacteriol. 101:333-338.

107. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208-218.

108. Marmur, J. and P. Doty. 1959. Heterogeniety in deoxyribonucleic acids. I. Dependence on composition of the configurational stability of deoxyribonucleic acids. Nature 183:1427-1429.

109. Marmur, J. and P. Doty. 1961. Thermal renaturation of deoxyribonucleic acids. J. Mol. Biol. 3:585-594.

110. Marmur, J. and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5:109-118.

111. Marmur, J. and D. Lane. 1960. Strand separation and specific recombination in deoxyribonucleic acids: Biological studies. Proc. Natl. Acad. Sci. U.S.A. 46:453-460.

112. Marmur, J., S. Falkow, and M. Mandel. 1963. New approaches to bacterial taxonomy. Annu. Rev. Microb. 17:329-372.

113. Marmur, J., R. Round, S. Falkow, L. S. Baron, C. Schildkraut, and P. Doty. 1961. The nature of intergeneric episomal infection. Proc. Natl. Acad. Sci. U.S.A. 47:972-979.

114. Mayer, L. W. and F. E. Young. 1978. Genetic transfer from Bacteroides fragilis to Escherichia coli. Abstr. Annu. Meet. Am. Soc. Microbiol. D-28, p. 36.

115. Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127:1529-1537.

116. Mitshuhushi, S. 1960. Drug resistance and its heredity in enteric bacteria. Kagaku (Tokyo) 30:628-633. [quoted by Watanabe (169)].

117. Mitshuhushi, S., K. Harada, and H. Hashimoto. 1960. Drug-resistance of enteric bacteria. IV. Generality of the appearance of multidrug-resistant enteric bacteria and experimental approach to obtaining multidrug resistant E. coli. Med. Biol. (Tokyo) 55:157-160. [quoted by Watanabe (169)]

118. Mitshuashi, S., K. Harada, M. Kameda, M. Suzuki, and R. Ezawa. 1960. On the drug-resistance of enteric bacteria. 3. Transmission of the drug-resistance from Shigella to F⁻ or Hfr strains of E. coli K-12. Jap. J. Exptl. Med. 30:301-306. [quoted by Watanabe (169)].

119. Moore, R. L. 1974. Nucleic acid reassociation as a guide to genetic relatedness among bacteria. Current Topics in Microbiol. and Immunol. 64:105-128.

120. Moore, W. E., E. P. Cato, and L. V. Holdeman. 1978. Some current concepts in intestinal bacteriology. The Am. J. Clin. Nutr. 31:S33-S42.

121. Moore, W. E. C. and L. V. Holdeman. 1973. New names and combinations in the genera Bacteroides Castellani and Chalmers, Fusobacterium Knorr, Eubacterium Prevot, Propionibacterium Orla-Jensen, and Lactobacillus Beijerinckii. Int. J. Syst. Bacteriol. 23:69-74.

122. Moore, W. E. C. and L. V. Holdeman. 1974. Human fecal flora: The normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. 27:961-979.

123. Nisloka, T., M. Mitani, and R. C. Clowes. 1969. Composite circular forms of R-factor deoxyribonucleic acid molecules. J. Bacteriol. 97:376-385.

124. Novick, R. 1969. Extrochromosomal inheritance in bacteria. Bacteriol. Rev. 33:210-325.

125. Novick, R. P. and D. Bouanehaud. 1971. Extrachromosomal nature of drug resistance in Staphylococcus aureus. Annu. N.Y. Acad. Sci. 182:279-294.

126. Novick, R. P., R. C. Clowes, S. N. Cohen, R. Curtiss III, N. Data, and S. Falkow. 1976. Uniform nomenclature for bacterial plasmids: A proposal. Bacteriol. Rev. 40:168-189.

127. Novick, R. P., K. Smith, R. J. Sheehy, and E. Murphy. 1973. A catenated intermediate in plasmid replication. Biochem. Biophys. Res. Commun. 54:1460-1469.

128. Novick, R., D. Zouzias, and M. Rush. 1975. Nucleic acid hybridization analysis of an integrated plasmid in Staphylococcus aureus. J. Bacteriol. 124:1424-1428.

129. Nygaard, A. P., and B. D. Hall. 1963. A method for the detection of RNA-DNA complexes. Biochem. Biophys. Res. Commun. 12:98-104.

130. Ochiai, K., T. Yamanaka, K. Kimura, and O. Sawacha. 1959. Studies on inheritance of drug resistance between Shigella strains and Escherichia coli strains. Nippon Iji Shimpo 1861:34-36 [as quoted by Watanabe (169)]

131. Orshov, I. and F. Orskov. 1966. Episome-carried surface antigen K-88 for Escherichia coli. I. Transmission of the determinant of the K-88 antigen and the influence on the transfer of chromosomal markers. J. Bacteriol. 91:69-75.

132. Pakroppa, W., W. Gocbel, and W. Muller. 1975. Isolation of closed-circular duplex DNA by chromatography on hydroxyapatite in the presence of ethidium bromide. Anal. Biochem. 67:372-383.

133. Palchaudhuri, S. and A. Chakrabarty. 1976. Isolation of plasmid deoxyribonucleic acid from Pseudomonas putida. J. Bacteriol. 126:410-416.

134. Podhairsky, C. and L. Reinhold. 1970. Bacteriocinbildung und Bacteriocin sensibilitat ber Bacteroides fragilis und Bacteroides thetaiotaomicron aus Klinschen material. Zentralbl. Bakteriol. Parasitenkd Infektionsk. Hyg. Abt. I Orig. 215:475-482.

135. Porter, R. W., R. Kolodner, and R. Werner. 1973. Plasmids of Shigella dysenteriae Y6R: a defective Col factor. J. Bacteriol. 116:163-174.

136. Prevot, A. R. 1938. Etudes de systematique bacterienne. Invalidite du genre Bacteroides (Castellani and Chalmers) demembrement et classification. Ann. Inst. Pasteur Paris 69:285-307.

137. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: The closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. 57:1514-1521.

138. Reanney, D. 1976. Extrachromosomal elements as possible agents of adaptation and development. Bacteriol. Rev. 40:552-590.

139. Reeves, P. 1965. The Bacteriocins. Bacteriol. Rev. 29:24-45.

140. Reider, J. and F. Macrina. 1976. Plasmid DNA isolation in Streptococcus mutans: glycine-enhanced cell lysis. In H. M. Stiles, W. J. Loesche, and T. C. O'Brien (eds.). Proceedings of microbial aspects of dental caries. Special Suppl. to Microbiol. Abstracts, Vol. 3, Information Retrieval, Inc., Washington, D.C.

141. Reng, K. J., G. J. Miraglia, and D. W. Lambe. 1975. Evaluation of agents useful in the treatment of anaerobic bacterial infections. Abstr. Annu. Meet. Am. Soc. Microbiol. A-16, p. 3.

142. Roussel, A. F. and Y. A. Chabbert. 1977. Taxonomy and epidemiology of Gram-negative bacterial plasmids studied by DNA-DNA filter hybridization in formamide. J. Gen. Microbiol. 104:269-276.

143. Royer-Pokora, B. and W. Goebal. Plasmids controlling synthesis of hemolysins in Escherichia coli. Molecular Gen. Genetics 144:177-183.

144. Rubens, C., F. Heffron, and S. Falkow. 1976. Transposition of a plasmid deoxyribonucleic acid sequence that mediates ampicillin resistance: Independence from host rec functions and orientation of isolation. J. Bacteriol. 128:425-434.

145. Rush, M., R. Novick, and R. DeLey. 1975. Detection and quantitation of Staphylococcus aureus penicillinase plasmid deoxyribonucleic acid by reassociation kinetic. J. Bacteriol. 124:1417-1423.

146. Schildkraut, D., L. Marmur, and P. Doty. 1961. The formation of hybrid DNA molecules and their use in studies of DNA homologies. J. Mol. Biol. 3:595-617.

147. Schildkraut, C., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4:430-443.

148. Sciaky, D., A. L. Montoya, and M. D. Chilton. 1978. Fingerprints of Agrobacterium Ti plasmids. Plasmids 1:238-253.

149. Sebald, M. and G. Brefort. 1975. Transfert du plasmide tetracycline-chloramphenicol chez Clostridium perfringens. Compt. Rend. Acad. Sci. 281:317-319.

150. Seidler, R. J. and M. Mandel. 1971. Quantitative aspects of deoxyribonucleic acid renaturation: Base composition, state of chromosome replication, and polynucleotide homologies. J. Bacteriol. 106:608-614.

151. Seki, T., T. Oshima, and Y. Oshima. 1975. Taxonomic study of Bacillus by deoxyribonucleic acid-deoxyribonucliec acid hybridization and interspecific transformation. Int. J. Syst. Bacteriol. 25:258-270.

152. Shipley, P. L., C. L.Gyles, and S. Falkow. 1977. Characterization of plasmids that encode for the K88 colonization antigen. Inf. Immun. 20:559-566.

153. Silver, R. and S. Falkow. 1970. Specific labeling and physical characterization of R-factor deoxyribonucleic acid in Escherichia coli. J. Bacteriol. 104:331-339.

154. Silver, R. and S. Falkow. 1970. Studies on resistance transfer factor deoxyribonucleic acid in Escherichia coli. J. Bacteriol. 104:340-344.

155. Smith, H. W. 1974. A search for transmissible pathogenic characteristics in invasive strains of Escherichia coli: The discovery of a plasmid-controlled toxin and a plasmid-controlled lethal character closely associated, or identical with colicine V. J. Gen. Microbiol. 83:95-111.

156. Smith, H. W. and S. Halls. 1968. The transmissable nature of the genetic factor in E. coli that controls enterotoxin production. J. Gen. Microbiol. 52:319-334.

157. Smith, L. DS. (ed.). 1975. In The Pathogenic Anaerobic Bacteria. Charles C Thomas, Springfield, Ill.

158. Smith, M. J., R. J. Britten, and E. H. Davidson. 1975. Studies on nucleic acid reassociation kinetics: Reactivity of single-stranded tails in DNA-DNA renaturation. Proc. Natl. Acad. Sci. U.S.A. 72:4805-4809.

159. Stiffler, P. W., R. Keller, and N. Traut. 1974. Isolation and characterization of several cryptic plasmids from clinical isolates of Bacteroides fragilis. J. Inf. Dis. 130:544-548.

160. Swings, J. and J. DeLey. 1975. Genome deoxyribonucleic acid of the Genus Zymomonas Kluyver and Van Neil 1936: Base composition, size, and similarities, Int. J. Syst. Bacteriol. 25:324-328.

161. Tally, F., D. Snydman, S. Gorbach, and M. Malamy. 1978. Abstracts of 18th Interscience Conference on Antimicrobial Agents and Chemotherapy (ASM).

162. Telford, J., P. Boseley, W. Schaffner, and M. Bicnstiel. 1977. Novel screening procedure for recombinant plasmids. Science 195:391-392.

163. Tinnell, W. H. and F. L. Macrina. 1976. Plasmid DNA content of selected Bacteroides fragilis subspecies. Infect. Immun. 14: 955-964.

164. Van Embden, J. D. A., W. J. Van Leeuwen, and P. A. M. Guinee. 1976. Interference with propagation of typing bacteriophages by extrochromosomal element in Salmonella typhimurium bacteriophage type 505. J. Bacteriol. 127:1414-1426.

165. Veillon, A. and A. Zuber. 1898. Sur quelque microbes strictment anaerobies et leur role en pathologie. Arch. d. med. exp. et d'Anat. 10:517-545.

166. Vinograd, J., and J. Lebowitz. 1966. Physical and topological properties of circular DNA. J. Gen. Physiol. 49:103-125.

167. Walker, C. B. and T. D. Wilkins. 1976. Use of semisolid agar for initiation of sure Bacteroides fragilis infection in mice. Inf. Immun. 14:721-725.

168. Warner, R., M. Rogolsky, B. B. Wiley, and L. A. Glasgow. 1975. Isolation of extrachromosomal deoxyribonucleic acid for exfolative toxin production from phage group II Staphylococcus aureus. J. Bacteriol. 122:99-105.

169. Watanabe, T. 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. 27:87-115.

170. Watanabe, T. and T. Fubasawa. 1960. Episomive resistance factors in Enterobacteriaceae. I. Transfer of resistance factors by conjugation among Enterobacteriaceae. Med. Biol. (Tokyo) 56:56-59 [quoted by Watanabe (169)]

171. Watanabe, T. and T. Fukasawa. 1960. Episomic resistance factors in Enterobacteriaceae. III. Elimination of resistance factors by treatment with acridine dyes. Med. Biol. (Tokyo) 56:71-74 [quoted by Watanabe (169)]

172. Watson, J. (ed.) 1976. Molecular Biology of the Gene. 3rd ed. Benjamin Press Inc., Menlo Prk, Ca. p. 222-224.

173. Watson, B., T. C. Cursier, M. P. Gordon, M. D. Chilton, and E. W. Nester. 1975. Plasmid required for virulence of Agrobacterium tumefaciens. J. Bacteriol. 123:255-264.

174. Welch, R. and F. Macrina. 1978. Transfer of multiple antibiotic resistance between strains of Bacteroides fragilis. Proc. Va. Acad. Sci., in press.

175. Werner, H. 1974. Differentiation and medical importance of saccharolytic intestinal bacteroides. Arzneimittel-Forschung Arneim-Forsch. 24:340-343.

176. Wetmur, J. 1976. Hybridization and renaturation kinetics of nucleic acids. Ann. Rev. Biophys. Bioeng. 5:337-361.

177. Wetmur, J. G. and N. Davidson. 1968. Kinetics of renaturation of DNA. J. Mol. Biol. 31:349-370.

178. Willetts, N. 1972. The genetics of transmissible plasmids. Annu. Rev. Genet. 6:257-268.

179. Zaenen, I., N. Van Lorebeke, H. Teuchy, and M. Van Martagu. 1974. Supercoiled circular DNA in crown-gall inducing Agrobacterium strains. J. Mol. Biol. 86:109-127.

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PLASMID DEOXYRIBONUCLEIC ACID OF STRAINS OF THE SACCHAROLYTIC INTESTINAL *BACTEROIDES*

by

Thomas Dale Mays

(ABSTRACT)

A collection of 120 strains of saccharolytic intestinal *Bacteroides* was examined for plasmid deoxyribonucleic acid (DNA). These strains were previously characterized for standard phenotypic properties (variety of carbohydrates fermented, metabolic end products, and complex carbohydrate hydrolysis). Additional phenotypic characterizations included bacteriocin production, cell wall analysis, and antibiotic susceptibilities. The interrelatedness of the strains had been determined by DNA homology experiments. The strains were distributed among 10 major DNA homology groups or genospecies.

Plasmid DNA was found in 52 strains (43%) distributed among all ten major DNA homology groups. The plasmids ranged in size from 2 to 65 megadaltons (Mdal) as estimated from their migration distance in agarose gels during electrophoresis. A small plasmid of 2 to 6 Mdal was found in 42 of the plasmid bearing strains. Seventeen of these strains also contained larger plasmids (20 to 65 Mdal). Ten strains contained only a large plasmid. There was no apparent correlation between the presence of any particular plasmid size with the DNA homology grouping of the host strains. Size estimations calculated from plasmid DNA reassociation curves ($C_o t$ curves) agreed with the agarose gel electrophoresis size determinations.

Four plasmids were selected for reference use in DNA homology studies. They were isolated from B. fragilis strains 2553 and 4076, B. thetaiotaomicron strain 5482, and B. uniformis strain T1-1 respectively. These plasmid preparations were radioactively labeled in vitro using the 4 deoxyribonucleic triphosphates (^{3}H labeled thymidine 5'-triphosphate) and Escherichia coli B polymerase I. The percent guanine plus cytosine content of two duplexed plasmid DNA preparations was determined by comparing their thermal stabilities with those of duplexed DNA from three bacterial strains whose percent guanine plus cytosine contents were known. Plasmid DNA p4076 and p5482 were found to have a percent guanine plus cytosine content of 39 and 43 respectively.

Three of the reference plasmids (p2553, p4076, and p5482) were large species (22 to 23 Mdal) and did not have any significant base sequence homology with one another. The small reference plasmid (pT1-1, 3 Mdal) had 30% DNA homology with the p4076 reference plasmid. Plasmid DNA preparations from all the plasmid carrying strains were allowed to renature with each of the labeled reference plasmids. Many of these plasmid DNA preparations contained multiple plasmid species. The pT1-1 reference plasmid had moderate (30 to 60%) or higher (61 to 100%) DNA homology with 35 of the plasmid DNA preparations. The reference plasmid DNA, p2553, had moderate or high homology with 10 unlabeled plasmid preparations while the p5482 reference plasmid had similar homology with 9. The p4076 reference plasmid did not have significant base sequence homology with any of the plasmid DNA preparations. Several strains contained plasmid DNA

that had homology with both pT1-1 reference plasmid and one (but not both) of the larger reference plasmids (p2553 and p5482). The homology between the unlabeled plasmid DNA preparations and the two reference plasmids may be due to either separate or hybrid plasmids. This was not determined.

A computer analysis of 81 phenotypic traits was made, comparing each with the plasmid DNA homology groups. No significant correlation was noted. Plasmid-mediation of bacteriocin production was considered, but there was no correlation between bacteriocinogenesis and the occurrence of any plasmid DNA homology group.