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Characterization of a Multiple Antibiotic Resistance Plasmid from *Proteus mirabilis*

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CHARACTERIZATION OF A MULTIPLE ANTIBIOTIC RESISTANCE PLASMID
FROM PROTEUS MIRABILIS

by

Camille Talanda Fath

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
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VITA

The author, Camille Talanda-Fath, is the daughter of Dr. and Mrs. Edmund Talanda. She was born in Milwaukee, Wisconsin on the 31st day of July in 1953.

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ABSTRACT

CHARACTERIZATION OF A MULTIPLE ANTIBIOTIC RESISTANT FACTOR FROM PROTEUS MIRABILIS

A Proteus mirabilis isolate associated with nosocomial urinary tract infection was obtained from the clinical microbiology laboratory at Hines Veterans Administration Hospital. The isolate, strain 2138, was resistant to at least ampicillin (Ap), carbenicillin (Cb), gentamicin (Gm), tobramycin (Tm), kanamycin (Km), neomycin (Nm), streptomycin (Sm), colistin (Cs), cephalothin (Cl) and tetracycline (Tc). Neutral sucrose gradient sedimentation of plasmid DNA isolated from strain 2138 revealed the presence of three size classes of plasmids, designated pFL10010, pFL10020, and pFL10030. The molecular weights of the plasmids, calculated from contour length measurements of electronmicrographs, were 90×10^6 , 5×10^6 , and 27×10^6 respectively. A resistance phenotype of Ap, Cb, Gm, Tm, Km, Sm and Cl was transferred to Escherichia coli J53-1 via conjugation with strain 2138. A single plasmid species having a molecular weight of 90×10^6 was isolated from the transconjugate J53-1 (pFL10010). Isolated plasmid species from strain 2138 were used to transform E. coli Cl200-1 to antibiotic resistance. No detectable transfer of antibiotic resistance occurred with the pFL10030 DNA, and only Ap and Cb resistance was transferred using pFL10020 DNA. Successful transfer of the resistance phenotype of Ap, Cb, Gm, Tm, Km, Sm, and Cl to strain

C1200-1 was achieved using pFL10010 DNA. Agarose gel electrophoresis of partially purified plasmid DNA from strain 2138 and the transformant C1200-1 (pFL10010) showed a single plasmid species in the transformant that co-migrated with pFL10010 from strain 2138. The host range of the pFL10010 plasmid was investigated. The plasmid was transferred by conjugation to several genera of the Enterobacteriaceae and to the non-Enterobacteriaceae species, Pseudomonas aeruginosa. However, the plasmid did not transfer to the unrelated species Agrobacterium tumefaciens, or to some of the Enterobacteriaceae species tested.

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CHAPTER I

INTRODUCTION

A. REVIEW OF RELATED LITERATURE

1. Historical Perspective

The discovery of the "miracle drug" (27) penicillin in 1941, and subsequent antibiotics in the last forty years has revolutionized medicine by drastically reducing morbidity and mortality from microbial disease (21). Unfortunately, the versatility of microorganisms in their adaptive capabilities has presented a formidable array of defenses against antibiotics. The acquisition of multiple antibiotic resistance by the Enterobacteriaceae and related gram-negative organisms has become a significant problem in modern medicine.

Knowledge of drug resistant microorganisms is as old as chemotherapy itself. In 1907, Paul Ehrlich (21) gave the first detailed report of Trypanosome brucei which became resistant to the trypanocidal activity of paraosaniline (arsenic) after repeated exposure to the drug. Subsequently, with the introduction of each anti-microbial agent came evidence of microbial resistance to its inhibitory actions.

Attempts to explain this phenomenon led to two prevalent theories. One view held that the resistant cells developed by spontaneous mutation independent of drugs and were selected by their use. The opposing view held that the resistant cells were the adaptive product of interaction between the drug and bacterial cell.

In 1943, Luria and Delbruck (64) established the fluctuation analysis which provided a statistical method to determine the probability distribution of the number of resistant bacteria to be expected on the hypothesis of mutation. Application of this method to penicillin resistance in Staphylococcus by Demerec (20) indicated that the resistance arose by spontaneous mutation where the drug acted as a selective agent. Further use of replica plating proved that drug resistant mutants appeared spontaneously before exposure to the drug, thus confirming the hypothesis of spontaneous mutation.

Outbreaks of Shigellosis in post war Japan challenged this theory. Initially, Shigella dysenteriae responded to sulfanalamide treatment, but the effectiveness of the drug waned after five years with the emergence of sulfanalamide resistant Shigella strains. The introduction of newer antibiotics including streptomycin, tetracycline and chloramphenicol, initially circumvented the problem. However, within seven years, clinical isolates were found to be resistant to each antibiotic, and some multiply resistant to all of the antibiotics used (110). Since the frequency of spontaneous mutation to resistance to one drug is small, 10^{-7} to 10^{-8} (21), and independent of that to another antibiotic, the frequency of spontaneous mutation to resistance to all four antibiotics simultaneously would be less than 10^{-28} (71). Given this probability, the occurrence of multiply resistant Shigella dysenteriae and Escherichia coli from the same patient led the Japanese microbiologists to the conclusion that sets of resistant genes were conferring resistance to several antibiotics simultaneously

and were being transferred en bloc between bacteria of the same and different species (106).

Genetic exchange among bacteria had been previously demonstrated (75). It had been established that bacteria could transfer genetic information by transformation (60) in which the deoxyribonucleic acid (DNA) extracted from cells of one strain could induce heritable changes in cells of another, transduction (78) in which bacterial DNA is carried by a phage vector to another cell, and conjugation (61) in which genetic material is transferred through direct cell to cell contact.

In 1959, Akiba and co-workers (1) and Ochiai and co-workers (85), independently demonstrated the transfer of multiple resistance traits from Escherichia coli to Shigella dysenteriae. In 1960, Mitsuhashi and associates (74) demonstrated that the drug resistance was not mediated by filterable agents, was independent of chromosomal transmission, and could be spontaneously lost. Watanabe and Fukasawa (112) confirmed that the transfer of drug resistance was mediated by cell to cell contact by use of the interrupted mating technique. These workers hypothesized that the resistance factors were carried on an episome termed the resistance transfer factor (RTF) having autonomous replication and being separate from F factors. The term R factor was proposed by Mitsuhashi in 1960 and was adopted to describe the postulated episomes. Recently, the suggestion was made to supplant this term with the more acceptable designation, R plasmid (83).

Simultaneous to the Japanese study came worldwide reports of R plasmids isolated in several gram-negative species resistant to

sulfanalamide, streptomycin, tetracycline and chloramphenicol and other antibiotics in an array of combinations. Occurrence of multiply resistant clinical isolates has increased steadily to become a major medical concern (74). These vast numbers of R plasmids have been given various designations. The term R plasmid is currently accepted to designate a plasmid that carries genetic information for resistance to antibiotics and/or other antibacterial drugs. A resistant donor is a bacterial strain that is capable of stably transferring resistant genes, usually by conjugation, to a recipient strain and is referred to as the R donor (83).

2. Physical Characterization of Plasmids

a. Chemical Composition

Plasmid molecules have been isolated and shown to consist of double stranded deoxyribonucleic acid (DNA)(23). Visualization with the electron microscope (111) has revealed that plasmids exist as covalently closed circular (CCC) DNA molecules (111). The molecular mass of plasmids may vary from less than 10^6 to greater than 10^8 daltons, with an average size of 6×10^6 daltons. R plasmids may carry as many as 100 genes, including traits other than drug resistance (8).

The inherent bouyant density of plasmid DNA, or mean base composition, is a consistent characteristic of a plasmid and can be used to separate plasmids of differing densities from each other (19, 66). A more specific measure of the evolutionary relatedness of plasmids can be determined by DNA-DNA hybridizations and heteroduplex analysis (47, 13). The latter method has been useful for mapping insertions,

deletions and duplications in plasmid DNA molecules (44).

5

b. Dissociation and Reassociation of Composite R Plasmids

Many R plasmids are composite structures consisting of resistance transfer factor (RTF) and resistance determinants (r-determinants) components, each of which may be capable of autonomous replication under certain growth conditions.

Anderson and Lewis (3) were the first to observe that R plasmids may exist as multiple replicons which can dissociate and reassociate depending upon host species and culture conditions. In their system, the transfer factor, designated Delta, was found to transfer independently of the resistance traits. When Delta was transferred into a cell having resistance traits to sulfonamides and streptomycin, but lacking transfer capability, the plasmids reformed and transferred both Delta and the resistance traits to a recipient. This provided the basis for the resistance mobilization test Anderson developed to detect the presence of transfer factors in other wild type strains (2).

Anderson (2) classified plasmids into two groups: Class 1 and Class 2, which were also designated by Clowes (73) as plasmid co-integrate and plasmid aggregate, respectively. In Class 1 plasmids, the resistance determinants and the transfer factor form a covalently bonded complex which is maintained and transferred as a single linkage group. In Class 2 plasmids the resistance determinants and the transfer factor exist as discrete plasmids, but are transferred together by an unknown mechanism, probably other than covalent linkage.

Guerry et al. (34) demonstrated that some plasmids were capable of both Class 1 and Class 2 transfers and that the host plays a significant role in plasmid organization.

The Anderson model proved inadequate in explaining the molecular nature of dissociated plasmids found in other species of Enterobacteriaceae, and there remains a controversy surrounding the proposed models for dissociation. The plasmid R-NR1 (also designated R-222 and R-100) confers resistance to sulfonamides, streptomycin, ampicillin and tetracycline. Analysis has shown the plasmid isolated from E. coli to have a molecular mass of 60×10^6 daltons and a bouyant density of 1.711 g/cc. However, when isolated from Proteus mirabilis this is not the case.

In the P. miribilis host, multiple R plasmid DNA bands were observed in cesium chloride density gradients when host cells were cultured in drug-containing media (73). Rownd (93) has shown that the molecular mass, density and copy number of plasmids depend upon the conditions under which the Proteus cells are cultured. When cultured in a drug-free medium, R-NR1 DNA propagated in Proteus forms a single satellite band with a density of 1.712 g/cc and a mass of 63×10^6 daltons. After prolonged growth of cells in medium containing any of the drugs to which R-NR1 conferrs resistance, except tetracycline, a band of greater density, 1.718 g/cc, is observed. If the plasmid DNA is isolated during transition between these two density states, a complex of molecules having a broad spectrum of densities between 1.712 and 1.718 g/cc was observed.

Proteus mirabilis containing a segregant of R-NR1 was found to be resistant to tetracycline but not to any other drugs. Since the trait was transmissible it was thought to contain the transfer factor linked to the tetracycline gene and was designated RTF-Tc (95). When grown in medium containing tetracycline this strain revealed a single satellite in cesium chloride gradients having an intrinsic bouyant density of 1.710 g/cc and mass of 49×10^6 daltons (50). Isolation of the segregant from cultures in back transition, i.e., shift from antibiotic medium to antibiotic-free medium, showed two peaks in cesium chloride gradients corresponding to densities of 1.710 and 1.718 g/cc. Table I summarizes the data.

Rownd (92) proposed the following model to explain the phenomenon. The R-NR1 plasmid consists of two component parts: the transfer factor linked to the tetracycline determinant (RTF-Tc) and the r-determinants. In drug-free medium, R-NR1 exists as a single species having a molecular mass of 63×10^6 daltons. In drug-containing medium, there is a selection for cells having a higher level of drug resistance as these would grow faster. The r-determinants are amplified by the dissociation of the composite R plasmid into individual replicons. The amplified r-determinants exist as poly-r-determinants joined in tandem repeats (89), which combine with a single RTF-Tc to form poly-r-determinant R plasmids. The broad intermediate density band found in back transition is indicative of varying copies of r-determinants per molecule. The small plasmid (14×10^6 daltons) seen in back transition represents a single r-determinant as would be

Table 1.

Molecular properties of plasmid R-NR1 from cells cultured in different media.^a

Plasmid	Culture Conditions	CsCl Gradients Satellite Bands	Bouyant Density (g/cc)	Countour Length (μ m)	Molecular Mass (daltons)
NR1	antibiotic free	1	1.712	37.0	63×10^6
NR1	antibiotic	1	1.718	8.2 - 42.0	$14-87 \times 10^6$
NR1	back transition ^b	2	1.710	28.7	49×10^6
			1.718	8.3	14×10^6
RTF-Tc	antibiotic free	1	1.710	28.7	49×10^6

^a Data from Hashimoto and Rownd (36).^b Cells originally grown in antibiotic containing medium then placed in antibiotic-free medium.

predicted from differences between the composite R-NR1 and the RTF-Tc molecular masses.

Falkow (23) contends that the large poly-r-determinants exist separately more often than in tandem with the RTF-Tc and account for the 1.718 g/cc peak in cesium chloride gradients and that during stationary phase in Proteus, R-NR1 exists as a dissociated molecule. Cohen and Miller (12) support this view. However, all investigators unanimously agree that R-NR1 can dissociate in Proteus hosts into separate units of replication, that the smaller units contain most of the drug resistance genes present in multiple copies, and that the RTF linked to the Tc resistance gene is present in only a few copies.

The transition of R-NR1 has not been observed in E. coli harboring the plasmid which is resistant to very high concentrations of drugs. However, an increased number of copies of monomeric composite R-NR1 plasmids per cell were found (95). Transition of R plasmids does not occur with most species and seems to be a peculiarity of the host species which harbors the plasmid (34). The behavior of R-NR1 in P. mirabilis is typical of several other R plasmids found in this species.

c. Plasmid Copy Number

The number of copies of a plasmid per cell (copy number) is usually estimated by comparing the ratio of plasmid DNA to chromosomal DNA with the ratio of their respective sizes (molecular masses) (94). There is usually a constant ratio of plasmid DNA to chromosomal DNA throughout the exponential phase of growth which indicates that there

is a fixed number of rounds of plasmid replication each division cycle (50). The number of rounds of plasmid replication is a characteristic of each type of plasmid and is one of the features of the control of its replication. In order for a plasmid to be maintained stably in a population of cells, there must be at least one round of plasmid replication during each division cycle. Many plasmids are under stringent control, that is, only one to two copies exist per chromosome; others are under relaxed control having several copies per chromosome.

By selecting for increased levels of drug resistance, it has been possible to isolate R plasmid mutants which have an increased number of copies per cell. These mutants have been referred to as round of replication (ROR) mutants, since they undergo an increased number of rounds of replication during the bacterial division cycle (55). These ROR mutants have the same size as the wild type R plasmids, showing that the number of copies of the drug resistance genes has not been increased by the formation of multimeric structures, as in transition in P. mirabilis (50).

d. Insertion Sequences

Insertion sequences (IS) are specific nucleotide sequences, of 800 base pairs or more, which have the ability to insert or excise from a segment of DNA. Initially discovered as "hotspots" for recombination (55), they provide a recognition site or area of homology between two genetic elements allowing for interaction. These sequences (IS-1, IS-2, IS-3, etc.) have been located at the interfaces

between the RTF and r-determinants of R-NR1 and several composite R plasmids (44). This suggests that recombination between IS elements may provide the mechanism by which an R plasmid can dissociate into its individual replicons (90).

e. Transposition

Mobility has been associated with several drug resistance markers. Foster and co-workers (28) attempted to introduce two incompatible plasmids, one conferring resistance to chloramphenicol but not to tetracycline and the other with opposite traits, into a single bacterium by selecting for transconjugates resistant to both antibiotics. The stable derivatives that were isolated were found to carry the tetracycline marker on the bacterial chromosome. The unit carrying the resistance marker was flanked by two inverted IS-3 elements (28). The tetracycline marker comprised a mobile determinant displaying the capacity for insertion and excision which was formerly thought to be a property of the insertion sequences themselves (40). This capacity has been described as translocation or transposition, and the unit that is transferred has been termed a translocon or transposon. The tetracycline transposon has been designated Tn9 (formerly TnD). Transposition has been shown to be independent of the cellular recombination (rec) systems (5, 57, 100).

Subsequently, several transposons have been identified (90). A penicillinase producing transposon, Tn1, has been found in several R plasmids. Heteroduplex mapping has shown Tn1 to be flanked by inverted repeats of less than 100 base pairs (40). A transposon, Tn9,

coding for chloramphenicol deactivation has been isolated in which no inverted repeats were detected. It has been suggested that transposition may not necessarily involve IS elements (83).

It is not known if there is a common mechanism or a variety of mechanisms for transposition of Tn and IS elements. The functional significance of the inverted repeats has not been elucidated, although it has been suggested that they are a stabilizing factor (63). Evidence does imply that insertion is not random. However, selectivity may be low, and excision has been known to cause deletions (41). Mutagenesis of bacterial and plasmid genes by insertions of transposons has also been demonstrated (90). The role of these genetic elements in the evolution and reassortment of R plasmids is thought to be significant (55). The use of antibiotics may create selective pressures that encourage the transposition of Tn-like elements onto transfer factors, allowing for construction and evolution of the composite R plasmid. A plasmid may acquire several r-determinants in this manner becoming a multiply resistant plasmid.

3. Genetic Characterization of Plasmids

a. Replication

The most important property of an R plasmid is its ability to replicate autonomously in the cytoplasm of the host cells. The autonomy of the plasmid has been demonstrated by isolation of discrete plasmid molecules. Inhibition of plasmid replication achieved by use of acridine dyes which do not affect cell growth provides convincing evidence of the independence of plasmid replicons (113).

Although plasmid replication is physically separate from the host chromosome, the limited number of plasmid molecules per host chromosome (109) and the unique time in the cell cycle of plasmid replication (117) suggest that cellular control exists. Rownd (50) has shown that the number of plasmids per chromosome (copy number) varies with host bacteria. The plasmid R-NR1 in E. coli exhibits stringent control (single copy) while in P. mirabilis relaxed control (multicopy) is observed (79). Chromosomal mutations making plasmid replication temperature sensitive have been characterized and support the view that there exist cellular controls on independent plasmid replicons (114).

Studies on plasmid replication indicate that one or more proteins are required for the initiation of R plasmid replication, and that a small pool of "initiator protein" is present in the cells during exponential growth. Some plasmids require ribonucleic acid (RNA) synthesis for replication while others, including R-NR1, do not (95).

b. Incompatibility

The introduction of a second plasmid into a plasmid bearing host may lead to the elimination of one plasmid, or segregation of the plasmids in subsequent generations (19). This phenomenon of mutual exclusion is shown by most known plasmids regardless of copy number and is a manifestation of relatedness of plasmids. This situation has permitted the assignment of plasmids to incompatibility sets, of which there are at least 26 among the enteric bacteria and 11 among those of Staphylococcus aureus (84).

Early models for incompatibility postulated that there is a specific membrane attachment site essential for plasmid replication, similar to that of the chromosome (14), and that competition for that attachment site may be coupled with production of a specific inhibitor of replication (43). Novick and Hoppensteadt (84) consider incompatibility to be a complex phenomenon involving several different plasmid genetic systems that either influence or determine autonomous incompatibility and its specificity. These determinants include the replication origin, the mechanism controlling copy number and the putative recognition system for partition (which may be considered as a centromere analog). There may be others, as yet unidentified.

According to Datta (16) plasmids are said to be incompatible if a host cell harboring a plasmid is immune to infection with a second plasmid. Compatibility between the two is indicated by retention by the host of both incoming and resident plasmids. Incompatibility is a transitive phenomenon i.e., if A is incompatible with B, and B with C, then A is incompatible with C. However, compatibility is not a transitive phenomenon (84). The system of classification into incompatibility groups has become inadequate due to difficulties in establishing experimental standard recipients, lack of distinguishable genetic markers on some plasmids and plasmid-plasmid interactions (31).

Interestingly, a correlation has been demonstrated between incompatibility grouping and DNA relatedness. Plasmids within an incompatibility group almost always show extensive DNA homology, whereas

plasmids of different groups rarely show significant homology (24). Although exceptions exist (24), extensive homology exists between plasmids of incompatibility group W (98) and within other groups. All the plasmids of this group determine different resistant patterns, were detected in different bacterial genera and were isolated in different parts of the world. The DNA of each hybridized with that of the others to at least 75% of its total length. Electron microscopy of heteroduplex molecules of these plasmids and their recombinants and deletion mutants, have permitted the identification of the DNA loci encoding the various resistance genes (98).

c. Chromosomal Integration

Although plasmids exist as extrachromosomal elements independent of the host chromosome, some, termed episomes, have the ability to integrate into the chromosome (74). This ability is host specific. For example, some plasmids behave as episomes in E. coli, but not in P. mirabilis (80). Some plasmids, once integrated, are able to excise from the chromosome. Abnormal excision may explain the presence in plasmids of genes governing host cell functions such as lac genes found in a Proteus plasmid (103).

d. Plasmid-Plasmid Interactions

Crossing over and recombination occur between different plasmids allowing genetic exchanges and plasmid-plasmid interaction. Recombinant plasmids have also been created in vitro by use of restriction endonucleases (105). These can be reintroduced into the host bacteria by the transformation process. Although it is presumed that

replication and integration of plasmids is similar to models for the chromosome, the molecular mechanism has not been fully elucidated.

e. Transmissibility

The ability of R plasmids to spread throughout a bacterial population is a major medical problem. Conjugal transmissibility occurs in three steps (76). First, the production of the pilus (conjugal bridge) is thought to provide the attachment site between donor and recipient cell. Following the formation of the initial specific contacts, many donor and recipient cells form wall to wall contacts (76). Second, the formation of specific contacts is thought to trigger a set of metabolic events that lead to the exit of DNA from the donor. These events prepare the DNA for exit and are termed mobilization. There has been question as to the function of the pilus. Some workers believe it to be merely a means of bringing the cells into contact and not used for passage of DNA (111). Others believe that the DNA passes through the pilus to the recipient cell. Regardless, the DNA is transferred as a single linear strand which must replicate and circularize in the recipient cell. Lastly, the genetic expression of the R plasmid phenotype in the recipient must occur. Interestingly, bacteriophages have been isolated which adsorb only to certain conjugation pili (68).

In addition to transmissible R plasmids, a large number of R plasmids have been isolated in the Enterobacteriaceae and S. aureus which cannot mediate their own transfer from cell to cell. These non-transmissible R plasmids usually confer resistance to one or two drugs,

are present in multiple copies per cell, and have molecular masses of only 5-10 megadaltons (95). Several bacterial genera also harbor transfer factors (sex factors) which do not carry drug resistance genes. Nontransmissible R plasmids can be mobilized (transferred to a recipient cell) by sex factors, transfer factors, or by transmissible R plasmids when both are present in the same host cell (2). The mechanism of transfer appears to involve covalent linkage between the non-transmissible plasmid and the transfer factor. Transfer factors and small nontransmissible R plasmids can co-exist within the same host and not recombine, as is true for any two compatible plasmids (95).

f. Host Range

Certain plasmids have the ability to infect a variety of unrelated bacterial strains, other plasmids are limited to a single host strain or related host species. The R plasmids found among the Enterobacteriaceae and related gram-negative species generally are transferable within the group. The frequencies of R plasmid transfer differ considerably among the species and depend upon the specific donor and recipient cells, mating conditions, and origin of the plasmid. Generally, E. coli, Shigella, Citrobacter and Klebsiella Enterobacter species are useful donors and recipients. However, species of Proteus, Salmonella and Serratia generally do not transfer plasmids at high frequencies in mating experiments (22).

g. Transduction

Generalized transduction provides an additional way of transferring genetic material from one bacterial parent to another. The agents active in transduction are phage-like particles that have en-

capsulated a fragment of bacterial DNA (81, 99). The significance of plasmid transduction in nature is uncertain, but it provides a useful genetic tool. In some cases all of the resistance traits and transmissibility can be transduced by phage, while, in other cases, only certain genes are transduced. The size of the DNA molecule that can be incorporated into the phage capsule probably determines the number of plasmid genes which are transduced. This phenomenon of "transductional shortening" has been useful in determining the genetic sequence of some plasmids (99).

In the transduction of an R plasmid conferring resistance to sulfonamide, streptomycin, tetracycline and chloramphenicol to Salmonella typhimurium using phage P-22 as vector, transductants received either sulfonamide-streptomycin-chloramphenicol resistance en bloc or tetracycline alone. Only in the latter case could the resistance then be transmitted through conjugation (108). In later experiments with the same plasmid the resistant traits were segregated in the transductants allowing for the construction of a genetic map of the plasmid (110). This indicated that the resistance genes are indeed different and that multiple drug resistance is not some pleiotrophic effect of a single gene. The most important conclusion is that the genes determining resistance and those determining conjugal ability are distinctly separable.

4. Molecular Basis of Drug Resistance

Bacterial resistance to a particular antibiotic or related series of antibiotics depends on a single mechanism, although occasionally a

combination of mechanisms may be found in the same organism (78). The most frequently found mechanism of resistance will be discussed here.

a. Beta-Lactam Resistance

The most prevalent mechanism of resistance is conversion of an active drug to an inert product by an enzyme found only in resistant bacteria. The existence of genes coding for drug inactivating enzymes in R plasmids of Enterobacteriaceae was first demonstrated for enzymes hydrolyzing the beta-lactam drugs (17). A single predominant plasmid-associated beta-lactamase was found in gram-negative R plasmids of wide taxonomic and geographical range, and was termed "TEM-like" beta-lactamase (54).

In terms of physical properties, substrate specificity and specific activity, most beta-lactamases fall into two general types: the TEM type (also called type 1) and the O type (also called type 2). The former type has a broader general activity on penicillins and cephalosporins, but considerably less on oxacillin. The latter has the opposite effect (39). The TEM type may be coded for by chromosomal or plasmid genes and is associated with transposons. The O type has only been found associated with plasmids (22).

These beta-lactamases are capable of hydrolyzing the beta-lactam ring of antibiotics rendering them inactive. In the gram positive bacteria the synthesis of beta-lactamase is induced by the presence of the antibiotic and the enzyme is excreted, inactivating its substrate extracellularly (17). Conversely, gram-negative cells synthesize the enzymes constitutively, but in smaller quantities than gram-

positive cells, and the enzyme remains periplasmic (17).

A second, less common family of penicillin-inactivation enzymes has been characterized. The enzyme, an amidase, inactivates the drug by hydrolyzing the CO-NH bond between the side chain and the 6-amino group in the penicillin acid residue (22). Enteric bacteria have been found to elaborate this enzyme, but R plasmid mediated resistance to penicillin has been solely associated with beta-lactamase activity (54).

b. Chloramphenicol Inactivation

The mechanism of R plasmid mediated chloramphenicol resistance involves a drug-inactivation enzyme that catalyzes acetylation of chloramphenicol in the presence of acetyl-coenzyme A. The vast majority of R plasmid-containing clinical isolates that are chloramphenicol resistant have been found to elaborate this constitutive enzyme, chloramphenicol acetyl transferase (45). Thus far the enzyme has appeared to be reasonably homogeneous in all isolates examined regardless of their bacterial host and geographical location (22). However, there are significant differences in enzyme levels observed among different R plasmid-containing chloramphenicol resistant isolates when the same R plasmid is transferred from host to host. As previously mentioned, transpositional activity has been associated with chloramphenicol resistance (28). This could well explain the observed homogeneity of chloramphenicol acetyltransferase activity found in the various unrelated species.

c. Aminoglycoside Resistance

The aminoglycosides constitute a large, diverse, but structurally related family of antibiotics. Gentamicin, tobramycin, streptomycin, kanamycin and other aminoglycoside antibiotics are still commonly employed against gram-negative infections. The mechanism of chromosomal resistance to streptomycin is associated with specific alterations of the target site of the antibiotic on the 30S ribosomal component. Such alterations prevent binding of the antibiotic (77). However, this type of resistance is rare among clinical isolates (22).

Studies on the biochemical mechanisms of R plasmid-mediated aminoglycoside resistance indicate that they are not associated with ribosomal alteration, but rather with a remarkable number of enzymes which modify the antibiotics (107). Despite the variety of enzymes, there are only three known classes of inactivating reactions. The antibiotics are inactivated by either N-acetylation, phosphorylation or adenylation of susceptible amino or hydroxyl groups, using adenosine triphosphate (ATP) or acetyl coenzyme A as cofactors (18). Most aminoglycoside antibiotics are subject to more than one inactivation reaction. The enzymes are constitutively synthesized and are retained within the bacterial cell (18).

Those enzymes involving phosphorylation of antibiotics are termed the aminoglycoside antibiotic phosphotransferase enzymes. Based on their physical properties and substrate specificity there are three groups of enzymes in this class. The first group called

the kanamycin phosphotransferases has three types: 1) the first inactivates kanamycin (Km), neomycin (Nm), ribostamycin (Rb), paromycin (Pm), and lividomycin (Lv); 2) the second inactivates Km, Nm, Rb, Pm and butarosin (Bt); 3) and the third inactivates Km, Rb, Bt and Lv. The second group contains only a single activity ribostamycin phosphotransferase, which inhibits Rb action exclusively. The third group contains two streptomycin (Sm) inactivating enzymes: Sm-3'-phosphotransferase and Sm-6'-phosphotransferase (78).

Acetylation of aminoglycoside antibiotics is carried out by the aminoglycoside antibiotic acetyltransferase enzymes. There are three major groups of enzymes in this category. The first is the Km-6'-N-acetyltransferase group of which four major types are known: 1) the first inactivates Km and Nm; 2) the second inactivates Km, Nm and gentamicin (Gm); 3) the third inactivates Km, Gm and 3'-4'-dideoxy kanamycin (Km-D); and 4) the fourth inactivates Km, Gm, Km-D and amikacin (Ak). The second group includes the aminoglycoside 3'-acetyltransferase I enzyme which inactivates Gm and sisomycin (Ss), and aminoglycoside 3'-acetyltransferase II which inactivates Gm, Ss, Km and tobramycin (Tm). The third group of acetylating enzymes contains aminoglycoside 2'-acetyltransferase which inhibits the action of Gm, Nm, Km, Km-D, Rb, Lv and Bt (78).

There are four groups of enzymes known that effect the adenylation of aminoglycoside antibiotics. These are called the aminoglycoside antibiotic adenylyltransferase enzymes. They are 1) Sm-adenylyltransferase which inactivates Sm and spectinomycin (Sp); 2) Sm-6'-adenylyltransferase which inactivates Gm, Km, Km-D and Tm;

3) Gm-Km-2'-adenylyltransferase which inactivates Gm, Km, Km-D and Tm; and 4) Tm-4'-adenylyltransferase which inactivates Tm, Km, Nm, Bt, Pm, and Ak (78).

d. Non-Enzymatic Resistance Mechanisms

Tetracycline resistance is probably the most common R plasmid-mediated drug resistance found in nature (21). Although the specific mechanism whereby tetracycline is inhibited is not known, the drug is probably blocked from entering the cell in sufficient concentrations to inhibit ribosomal function. The resistance determinant may code for a new membrane protein which will not bind tetracycline. Franklin and Cook (29) have shown that the decreased uptake of the drug, and concomitantly the level of resistance, is appreciably increased in the presence of tetracycline, suggesting that the inhibitor of tetracycline is inducible. There is no evidence for enzymatic inactivation or modification of tetracycline, the resistance appears to be associated with modification of cellular permeability of the drug.

Bacterial resistance to p-aminobenzenesulfonamide (sulfonamide: Sa) and its resistance has been described. Observations that the drug is neither destroyed nor modified by R plasmid-containing strains suggest that resistance is due to decreased permeability of the cell membrane to sulfonamide (107). Pato and Brown (88) have reported a second type of resistance mechanism involving the sulfonamide sensitivity of the enzyme dihydropteroate synthetase, which decreases in accordance with an increase in Sa resistance. Recent findings indicate that the most general mechanism of high Sa resistance in gram-negative bacteria carrying R plasmids is due to the production of Sa-

resistant dihydropteroate synthetase which differs from Sa-sensitive enzyme produced by sensitive strains in Sa-susceptibility and in various enzymological properties (78).

Bacterial drug resistance may be affected by changes in the target site of the drug as mentioned for streptomycin resistance (45, 77). Streptomycin resistance is caused by mutation of the S-12 protein of the ribosome which is the ultimate binding site for the antibiotic. It is uncertain whether the mutation is induced by the presence of the antibiotic. It may result from use of an alternate pathway selected after elimination of the primary pathway by streptomycin. Of clinical significance are two forms of antibiotic resistant enzymes, a naladixic acid resistant DNA gyrase and a rifampicin resistant RNA polymerase, both of which are examples of alterations of target sites of antibiotics (45).

5. Versatility of Plasmids

R plasmids are known which also mediate resistance to a number of heavy metals, and ultraviolet irradiation. Mercury resistance appears to be inducible and associated with enzymatic catalysis (104). The mechanism for resistance to other heavy metallic ions, such as nickel or cobalt, has not yet been elucidated (22).

Other plasmids are known which contain genetic determinants unrelated to those for antibiotic resistance. Col plasmids have been discovered which confer colicinogeny to Escherichia coli (42). Degradative plasmids found in some species of Pseudomonas have been characterized by their ability to code for enzymes that catabolize a

great variety of unusual organic compounds by special metabolic pathways (42). Plasmids have been described which contribute to the virulence of E. coli by coding for production of alphanhemolysins (Hly plasmids) (100), enterotoxins (Ent plasmids) and surface antigens (101). Many gram-negative species harbor plasmids which do not carry genes which can be phenotypically detected. These cryptic plasmids can only be detected through DNA isolation techniques (33).

6. Gram-Positive Bacterial Plasmids

Plasmids have been isolated from species of Staphylococcus aureus which produce beta-lactamase and other antibiotic inactivating enzymes (82). Although multiple-resistant strains have been isolated, it has been found that each resistant determinant is usually located on a different plasmid, and that the plasmids, in general, tend to be smaller than those found in gram-negative species (45). It was originally thought that Staphylococcal plasmids were non-conjugal (22), and although conjugation has been demonstrated it is considered a rare occurrence (48). The facts indicate that transduction with phage lysates is mainly responsible for acquisition of drug resistance and for the wide and rapid distribution of multiple resistance among Staphylococci (48). The plasmids may exist in high copy number, up to 25 copies per cell, which accounts for resistance to high levels of antibiotics (48).

Multiple resistance plasmids have been isolated from species of Streptococcus faecalis. These have been shown to effect their own transfer through the conjugation process. Additionally, intergenic

conjugal transmission has been demonstrated between the streptococci and Lactobacillus species (49). Conjugation among streptococcal species is no longer considered a rare occurrence (49).

B. INTRODUCTION TO THIS STUDY

The prolongation of life in persons with severe underlying diseases that affect host defenses combined with the increasing use of corticosteroids, immunosuppressive agents, cytotoxic drugs and antibiotics has resulted in the emergence of a relatively new spectrum of infectious diseases. Ubiquitous previously saprophytic commensals, rarely causing infection in the normal host, are now recognized with increasing frequency in patients with compromised defense mechanism (7). Infections with such organisms pose a dilemma not only in diagnosis, because of their relative newness, but in therapy due to their increased resistance to conventional antibiotic treatment (52).

The Center for Disease Control reported in 1978 that 3.6% of patients hospitalized in the United States acquired nosocomial infections. More than half of those infections either caused or contributed to the death of the patient (HEW Publication (CDC) No. 78-8257). Of the nosocomial infections that year, nearly half were caused by members of the Enterobacteriaceae and related bacterial species. These organisms, termed conditional pathogens, are usually present in normal flora and spread by human contact (98).

This study initially began as an investigation into the increasing frequency of nosocomial infections at Hines Veterans Hospital at Hines, Illinois (65). Several strains of Proteus mirabilis isolated from clinical specimens were found to be clinically resistant to

gentamicin and ampicillin which were commonly used in their treatment. One isolate, Proteus mirabilis 2138, was chosen as the subject for this study because of its resistance to these and several other antibiotics.

This study was undertaken to elucidate the basis of this multiple-antibiotic resistance, specifically to determine whether R plasmids were involved, and if so, whether they were capable of being transferred to other gram-negative conditional pathogens. The aims of this research are 1) to study the genetic nature of multiple antibiotic resistance in Proteus mirabilis 2138; 2) to characterize the molecular nature of that genetic determinant; and 3) to determine the transmissibility and host range of the antibiotic resistance pattern.

CHAPTER II

MATERIALS AND METHODS

1. Bacterial Strains

The bacterial strains used in this investigation are listed in Table II. *Proteus mirabilis* 2138 was isolated at Hines Veterans Administration Hospital at Hines, Illinois and found to be resistant to both gentamicin and ampicillin/carbenicillin in routine laboratory screening. With the exception of the *Agrobacterium tumefaciens* strains which were grown at 30 C, all bacteria were grown at 37 C. For the purpose of counter selection, spontaneous rifampicin resistant mutants of certain bacterial strains were isolated. This was achieved by innoculating sensitive cells on nutrient agar plates containing 5 µg/ml rifampicin. Surviving colonies were picked and restreaked onto plates containing progressively higher concentrations of the drug (10, 15, 50, 75, 100 and 200 µg/ml) until the clones were able to grow well on nutrient agar plates containing 200 µg/ml rifampicin. The resistant clones were maintained in this manner.

2. Chemicals

All chemicals used were of the highest quality available and not further purified. Radioactive compounds were purchased from New England Nuclear. Enzymes and amino acids were purchased from Sigma Chemical

Table II.

Standard bacterial strains used.

Strain ^a	Relevant characteristics ^b	Origin
<u>Escherichia coli</u>		
J53	K-12 <u>pro</u> <u>met</u> F ⁻ λ ⁺	S. Falkow
J53-1	K-12 <u>pro</u> <u>met</u> F ⁻ λ ⁺ Rm ^r	This labora- tory
C1200	C <u>his</u> <u>met</u> F ⁻ λ ⁻	E. W. Six
C1200-1	C <u>his</u> <u>met</u> F ⁻ λ ⁻ Rm ^r	This laboratory
CSH-K-12	Prototroph	M. Stodolsky
<u>Proteus mirabilis</u>		Hines
2138	Hospital isolate, prototroph	V.A. Hospital
Pml5	Leu ⁻ Trp ⁻ Thy ⁻ Gal ⁻ Nia ⁻	R. H. Rownd
<u>Agrobacterium tumefaciens</u>		
B6-806		This laboratory
15955		This laboratory
<u>Enterobacter cloacae</u>		
ATCC 23355	Prototroph	W. Yotis
<u>Klebsiella pneumonia</u>		
ATCC 27730	Prototroph	W. Yotis
<u>Proteus vulgaris</u>		
ATCC 6380	Prototroph	W. Yotis
<u>Serratia marcescens</u>		
ATCC 8100	Prototroph	W. Yotis
<u>Pseudomonas aeruginosa</u>		
PA038	<u>leu</u> -38 FP2 ⁻	R. Olsen
<u>Salmonella typhimurium</u>		
TAl637	LT2 <u>his</u> <u>ade</u>	H. W. Taber
<u>Shigella flexneri</u>		
SFl	Asp ⁻ Asn ⁻ Leu ⁻ Ile ⁻ Val ⁻ Trp ⁻ His ⁻ Nia ⁻	R. J. Christiansen

^a K-12, serotype K-12; C, serotype C.

^b Asn - asparagine, Asp - aspartic acid, His - histadine, Ile - isoleucine, Leu - leucine, Met - methionine, Pro - proline, Trp - tryptophan, Val - valine, Ade - adenine, Nia - niacin, Thy - thymidine and Gal - galactose.

Company, agarose (HGT) was purchased from SeaKem. Pharmaceutical companies provided many of the antibiotics used for this study.

3. Media

Complex media used were: nutrient agar (NA), containing per liter 8.0 g Nutrient Broth (Difco) and 12.0 g agar (Difco); L Broth, containing per liter 5.0 g yeast extract (Difco), 10.0 g tryptone (Difco) and 5.0 g NaCl; MacConkey's Agar (Difco); and Mueller-Hinton Agar (M-H) (Difco).

Minimal medium for Escherichia coli (EM) contained per liter: 3.0 g K_2HPO_4 , 1.0 g KH_2PO_4 , 5.0 g NH_4Cl , 1.0 g NH_4NO_3 , 2.0 g $NaSO_4$ (anhydrous) and 0.1 g $MgSO_4 \cdot 7H_2O$ (86).

Minimal medium for Pseudomonas aeruginosa strains (PM) contained per liter: 6.0 g Na_2HPO_4 , 2.0 g KH_2PO_4 , 0.1 g $MgSO_4 \cdot 7H_2O$, 1.0 g NaCl and 5.0 g $Na_3C_6H_5O_7$ (sodium citrate) (86).

Agrobacterium tumefaciens strains were grown in minimal medium which contained per liter: 1.0 g Na_2HPO_4 , 3.0 g KH_2PO_4 , 0.3 g $MgSO_4$, 1.0 g NH_4Cl , 0.2 g KCl, 0.2 g $CaCl_2$ and 0.1 g Fe_2SO_4 (62).

Minimal medium phosphate buffers and salts were made separately as 20X stock solutions which were autoclaved and added to sterile dH_2O to achieve 1X concentrations. Sterile glucose (50%) was added to achieve a final concentration of 0.2%. To sustain auxotrophic strains 50 $\mu g/ml$ of the appropriate amino acids, 25 $\mu g/ml$ nucleoside or 10 $\mu g/ml$ nicotinamide were added. Solidified minimal medium contained 1.2% agar.

4. Determination of Minimal Bactericidal Concentrations (MBC)

The minimal concentration of an antibiotic necessary to kill a suspension of cells (MBC) was determined by the tube dilution technique. Tubes containing 10 ml of serially diluted antibiotic (10^{-1} - 10^{-8}) in L broth were inoculated with 1 ml of a bacterial suspension (approximately 50 Klett units, Klett-Summerson Photoelectric colorimeter model 800-3, No. 66 red filter) and incubated overnight. A 0.1 ml sample from each tube was plated on NA and incubated overnight. The lowest dilution of antibiotic that yielded no growth on the NA plate was given as the MBC.

5. Determination of the Antibiotic Resistance Spectrum

The Kirby-Bauer disk diffusion technique (69) was used to determine the antibiotic resistance spectrum. Onto Mueller-Hinton plates confluent streaked with a bacterial suspension were placed disks containing antibiotics. The plates were incubated at 37 C for 24 h. Zones of inhibition of bacterial growth around each disk were measured and compared to standard charts relating zone diameter to antibiotic resistance. Results were recorded as resistant (R) or sensitive (S). Donor, recipient, transconjugate and transformant strains were tested for resistance to the following drugs: gentamicin (Gm), tobramycin (Tm), neomycin (Nm), kanamycin (Km), streptomycin (Sm), tetracycline (Tc), ampicillin (Ap), carbenicillin (Cb), cephalothin (Cl), colistin (Cs) and rifampicin (Rm).

6. Genetic Experiments

a. Conjugations

Precultures of donor and recipient cells in 10 ml L broth were grown overnight in a shaking incubator, diluted 1:10 with fresh L broth and grown to late exponential phase (Klett reading of 50-100). Conjugation mixtures consisted of 1.2 ml fresh L broth, 0.2 ml donor cells and 0.6 ml recipient cells. Controls included 1.8 ml fresh L broth plus 0.2 ml donor cells, and 0.6 ml recipient cells in 1.4 ml fresh L broth. The mating mixture and controls were incubated at 37 C with minimal agitation for 24 h. The titer of input donor cells was determined by plating serial dilutions in triplicate on NA plates. The plates were incubated for 24 h and the number of colonies counted.

The conjugation mixture and controls were centrifuged at 10,000 rpm for 10 min to pellet the cells which were then washed in minimal medium lacking glucose and recentrifuged. The washed cells were resuspended in minimal medium, diluted and plated on selective media. A 0.1 ml sample of the mating mixture and each control were plated in triplicate on selective media. Additionally, the donor, recipient and mixed cultures were each plated on minimal medium lacking antibiotics, minimal medium containing antibiotics and on nutrient agar. The plates were incubated until growth was apparent (1-5 days). If no growth appeared after 7 days, negative results were recorded.

Matings that failed to demonstrate detectable transfer of antibiotic resistance traits after three trials were repeated using

sterile nitrocellulose filter disks to concentrate the cells. Pre-cultures were grown and diluted and regrown as above, 5 ml aliquots of donor and recipient cells were concentrated by vacuum suction onto membrane filters (0.45 μ m pore size, Millipore, Inc.) which were transferred growth side up to NA plates. Donor and recipient cultures were filtered likewise as controls. Following overnight incubation the filters were placed in growth flasks containing 10 ml L broth and swirled to resuspend the cells. The cell suspensions were pelleted by centrifugation, washed twice in minimal medium lacking glucose and plated as previously described.

b. DNA-Mediated Transformations

The method of Lederberg and Cohen (60) was used to transform Escherichia coli strains with purified plasmid DNA. An overnight preculture of the recipient strain was used to inoculate 50 ml of fresh broth. The cells were grown to exponential phase, transferred to a sterile, chilled centrifuge tube and incubated on ice for 5 min. The cells were pelleted by centrifugation for 10 min at 10,000 rpm, the supernatant was discarded and the cell pellet resuspended in 25 ml of ice cold 0.1 M $MgCl_2$. The cells were transferred to a 30 ml centrifuge tube, iced for 20 min and pelleted by centrifugation as before. The cells were resuspended in 2.5 ml of ice cold 0.1 M $CaCl_2$ and transferred to a sterile test tube.

The transformation mixture contained 0.1 ml purified plasmid DNA (50 μ g/ml) in TEN buffer (0.02 M Tris-HCl, 0.001 M EDTA, 0.02 M NaCl, pH 8.0) to which 0.2 ml competent cells from above were added.

Controls had additional TEN buffer substituted for the DNA. The mixtures were iced for 30 min, and then placed directly into a 42 C waterbath for 2 min to enable uptake of DNA. To 9.9 ml of prewarmed L broth, 0.1 ml of the transformation mixture or control was added. These cultures were incubated with shaking at 37 C for 90-120 min to allow full expression of the antibiotic resistance trait to be selected. Appropriate dilutions of the mixtures were plated on selective media and incubated until colonies appeared. To check the sterility of the DNA and the viability of the cells, 0.1 ml of each was plated onto nutrient agar.

7. Determination of Plasmid Stability

To determine the stability of the plasmids in transconjugate strains, 100 clones from each mating were picked onto nutrient agar plates. The clones were passed daily onto fresh media. At two week intervals, the clones were picked onto two additional sets of plates containing either gentamicin (50 µg/ml) or carbenicillin (100 µg/ml). Those clones that grew on the nutrient agar plates, but failed to grow on the gentamicin or carbenicillin plates were retested to confirm their loss of resistance.

8. Radiolabelling of Bacterial DNA

Overnight precultures of cells grown in minimal medium were used to inoculate 30 ml cultures of minimal medium supplemented with 0.2% glucose, 0.5% casamino acids (Difco) and 300 µg/ml deoxyadenosine to enhance the incorporation of (³H)-thymidine (35). At Klett readings

of 10, 20, and 30, 0.3 ml of (^3H)-thymidine (New England Nuclear Corp., 40-60 Ci/mole, 1 mCi/ml) was added to the culture. The cultures were grown to a final Klett reading of 50-100 and the cells harvested by centrifugation at 10,000 rpm for 10 min (Sorvall RC-5 centrifuge, SS-34 rotor). The cells were resuspended in minimal medium lacking glucose, washed and centrifuged as before. The cells were lysed and the DNA isolated as described below.

9. Isolation of Extrachromosomal DNA

Several methods were used to isolate extrachromosomal DNA from Proteus mirabilis 2138 and Escherichia coli J53-1(pFL10010) strains. The methods vary according to the susceptibility of the bacterial cells to lysis and the size of the plasmid molecule being isolated. Cells were grown in L broth unless otherwise stated.

a. Helinski Cleared Lysis

In a typical preparation (58), a 30 ml bacterial culture grown at 37 C to a cell density registering between 50-100 Klett units was harvested by centrifugation, washed twice with cold (4 C) TES buffer (0.05 M NaCl, 0.005 M EDTA, 0.05 M Tris-HCl, pH 8.0) and pelleted by centrifugation at 4 C for 10 min at 10,000 rpm. The pellet was resuspended in 1 ml cold 25% sucrose in 0.05 M Tris-HCl, pH 8.0. After the addition of 0.2 ml of lysozyme (Sigma Chemical Co., 5 mg/ml in 0.25 M Tris-HCl, pH 8.0) the suspension was placed on ice for 5 min. Ethylenediamine-tetraacetic acid (EDTA, 0.4 ml, 0.25 M, pH 8.0) was added to the cells followed by 5 min of incubation at 4 C. Complete cellular lysis was accomplished by the addition of

1.6 ml of a detergent mixture containing 1% Brij 58 and 0.4% sodium deoxycholate in 0.0625 M EDTA, 0.05 M Tris- HCl, pH 8.0. After 5-10 min at room temperature the suspension became relatively clear and viscous and was placed in a 30 ml centrifuge tube and centrifuged for 25 min at 18,500 rpm (Sorvall RC-5 centrifuge, SS-34 rotor). The supernatant containing the plasmid DNA was carefully removed to minimize contamination with pelleted chromosomal DNA and subjected to cesium chloride-ethidium bromide (CsCl-EtBr) density centrifugation as described below.

b. Guerry Method

A modification of the Helinski method by Guerry et al. (35) was used to isolate plasmid DNA for agarose gel electrophoresis. Bacterial cells were grown, harvested and treated with lysozyme and EDTA as above. Sodium dodecyl sulfate (SDS) was added to a final concentration of 1% to achieve complete cellular lysis. After lysis, 4.0 M NaCl was added to the viscous solution with gentle mixing to achieve a final salt concentration of 1 M. The lysates were stored at 4 C overnight, after which they were centrifuged at 17,000 rpm for 30 min at -10 C. The aqueous phase was removed, doubled in volume by the addition of dH_2O and prepared for agarose gel electrophoresis as described below.

Plasmid DNA, partially purified by the Guerry method was prepared for agarose gel electrophoresis by the method of Meyers et al. (71). The supernatant was treated with 20 $\mu\text{g/ml}$ ribonuclease A (bovine pancreas, Sigma Chemical Co., 1 mg/ml in 50 mM sodium acetate

pH 5.0, heated at 90 C for 10 min) and incubated for 45 min in a 37 C waterbath. After RNase digestion, an equal volume of redistilled phenol saturated with 0.05 M Tris-HCl, pH 7.5 was added, phases were gently mixed and the emulsion centrifuged at 12,000 rpm for 20 min at 20 C. The aqueous phase was removed. If it appeared cloudy the phenol extraction was repeated. The interphases of all phenol extractions were pooled and again extracted with phenol, and the aqueous layers combined. An equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added, carefully mixed and centrifuged as before to obtain a clear aqueous phase. The partially purified plasmid DNA solution was brought to a final concentration of 0.3 M sodium acetate by the addition of one-tenth volume of 3.0 M sodium acetate solution, and twice the volume of cold (-20 C) 95% ethanol was added to precipitate the DNA. The mixture was well mixed and stored at -20 C overnight. The precipitated DNA was pelleted by centrifugation at 12,000 rpm, 20 min, at -10 C. The supernatant was decanted and the tube allowed to thoroughly drain. The DNA was redissolved in 0.1 ml of buffer containing 0.03 M Tris-HCl, 0.05 M NaCl, and 0.005 M EDTA, pH 8.0, and analyzed immediately by agarose gel electrophoresis or stored at -20 C until ready for use.

c. Currier Method

A modification of the Currier method (15) was used to isolate large plasmids. All steps were carried out at room temperature unless otherwise stated. An overnight preculture was used to inoculate 500 ml of L broth. Cells were grown to a density corresponding to a reading of 50-100 Klett units, and harvested by centrifugation. The

cells were washed twice in EM lacking glucose and resuspended in 49 ml of 0.05 M Tris-HCl, pH 8.0. After thorough mixing, 1.0 ml of 1.0 M EDTA, pH 8.0 was added to the cell suspension. A solution of lysozyme in TE buffer (0.05 M Tris-HCl, 0.02 M EDTA, pH 8.0) was added to achieve a final concentration of 200 g/ml and the mixture was incubated for 5 min in a 37 C waterbath. Carefully, 2.5 ml of 20X SDS solution was added to make 1% final concentration. Incubation at 37 C was resumed until a clear lysate of high viscosity was obtained (10-60 min).

The lysate was sheared until it flowed evenly by several passages through an 18 gauge hypodermic needle attached to a 50 cc syringe. The DNA was denatured by adjusting the pH of the sheared lysate to 12.1 - 12.4 by the dropwise addition of 3N NaOH with continuous stirring on a magnetic stir plate. After 10 min of mixing, the pH of the solution was brought to approximately 8.5 by the rapid addition of 2.0 M Tris-HCl, pH 7.0. Stirring was continued for 3 min. The lysate was adjusted to a final concentration of 3% (w/v) NaCl by the addition of solid salt. An equal volume of redistilled phenol equilibrated with 3X SSC (0.15 M NaCl, 0.015 M trisodium citrate) was added and the lysate mixture was stirred for 7 min fast enough to mix the phases. The lysate was centrifuged at 10,000 rpm for 10 min and the aqueous phase removed to a clean flask without disturbing the precipitate at the interphase. A second extraction using an equal volume of chloroform-isoamyl alcohol (24:1 v/v) proceeded likewise. To the aqueous phase a 0.02 volume of 1.0 M sodium phosphate buffer was

added followed by the addition of 0.025 volume of 1.0 M magnesium chloride. The solution was well mixed by swirling the flask. A 0.7 volume of cold (-20C) 95% ethanol was added while swirling the flask. If the solution did not become cloudy the magnesium chloride addition was repeated. The lysate was stored at -20C for 1-24 h. Precipitated DNA was collected by centrifugation for 10 min at 10,000 rpm, re-dissolved in a minimal volume (less than 5 ml) of 0.25 M EDTA, pH 8.0 and dialyzed against TES buffer for 24 h. The plasmid DNA was further purified by dye-bouyant-density centrifugation as described below.

Alternately, following the chloroform-isoamyl alcohol extraction, the aqueous phase was brought to a final concentration of 0.3 M sodium acetate by the addition of 0.1 volume of 3.0 M stock solution. Twice the volume of cold ethanol (-20C) was added to the solution which was stored at -20C overnight. The precipitated DNA was collected by centrifugation at 12,000 rpm for 20 min at -5 C. After allowing the centrifuge tube to drain, the pellet was resuspended in 2.0 ml TES buffer and prepared for CsCl-EtBr centrifugation.

d. Humphreys Method

Early in this work concentration of DNA from large lysates was achieved through use of polyethylene glycol (PEG) precipitation of cleared lysates (46) prepared from 500 ml cultures. After the addition of NaCl to achieve a final concentration of 0.5 M, the lysates were adjusted to 10% PEG (w/v). The mixture was dissolved by swirling the flask, covered and stored overnight at 4 C. Centrifugation at 10,000 rpm for 10 min pelleted the DNA. After discarding the supernatant,

the pellet was resuspended in a minimal volume of TES buffer and purified by CsCl-EtBr centrifugation.

10. Agarose Gel Electrophoresis

Plasmid DNA prepared by the Guerry-Meyers method described above, was subjected to electrophoresis in 0.7% agarose (SeaKem, Inc.) dissolved in Tris borate buffer (89.0 mM Tris base, 2.5 mM EDTA, 89 mM boric acid) (71). To each sample, 5 μ l of dye solution (0.07% bromophenol blue, 7.0% SDS, 33% glycerol in dH_2O) was added prior to electrophoresis. A horizontal slab gel apparatus (20 x 20 cm) with wells 1 mm x 12 mm x 7 mm deep was used. The electrophoresis was carried out at 60 mA, 120 V for 3-4 h or until the dye had travelled 10-12 cm. The gel was placed in a solution of ethidium bromide (0.5 $\mu\text{g/ml}$ in dH_2O) and stained for 15 min. The DNA bands, illuminated by a near UV (366 nm) transilluminator (UV Products, Inc.) were photographed with a Poloroid MP4 camera using type 57 film and UV 17 and type 21 filters.

11. Cesium-Chloride-Ethidium Bromide Density Centrifugation

Purification of plasmid DNA was achieved by subjecting the partially purified DNA to cesium chloride-ethidium bromide (CsCl-EtBr) equilibrium density gradient centrifugation (28, 132). In the dark, 7.0 ml of the DNA in TES buffer was added to 7.3 g CaCl. To this 0.6 ml EtBr (10 mg/ml in TES buffer) was added and the refractive index adjusted to 1.392. If PEG or sodium acetate was used to precipitate the DNA, the refractive index was adjusted to 1.3925 or 1.3940

respectively. The solution was placed in a polyallomar centrifuge tube (Beckman), topped with mineral oil, capped and centrifuged to equilibrium (48 - 72 h, Beckman L-4 or L-5-65 ultracentrifuge, 40 rotor, 35,000 rpm, 20 C).

Following centrifugation, bands of DNA were visualized using ultraviolet light. The larger upper band contained linear chromosomal DNA, and the smaller lower band contained the supercoiled plasmid DNA which bands at a greater density than the linear form. The plasmid DNA was removed either by side puncture of the tube using an 18 guage needle attached to a 3 cc syringe or by aspiration of the oil and upper band by mild vacuum and removing the remaining plasmid band using a pasteur pipette with the tip cut off. Ethidium bromide was removed by extracting the DNA solution several times with isopropyl alcohol saturated with 20X SSC. Cesium chloride was removed by overnight dialysis against TES buffer. In most cases the plasmid DNA was repurified by a second centrifugation in CsCl-EtBr.

Cesium chloride-ethidium bromide density gradients containing (^3H)-DNA were fractionated by bottom puncture of the centrifuge tube. Fractions containing 15-18 drops each (approximately 0.15 ml) were tested for the presence of radioactivity by spotting a 5 μl sample of each onto Whatman filter strips. The strips were dried, washed twice in 5% trichloroacetic acid (TCA) for 5 min, and twice in 95% ethanol for 5 min. The strips were cut to divide fraction samples and each was placed into a scintillation vial containing 10 ml scintillation cocktail (Toluene-Liquifluor, NEN). Radioactive counts

were determined in a Packard Tri-Carb Liquid Scintillation Spectrometer. When plasmid DNA was present peaks were seen. The first dense peak containing the plasmid DNA fractions was collected and subjected to a second CsCl-EtBr centrifugation for further purification.

12. Sucrose Gradients

a. Alkaline Sucrose Gradients

Solutions of (^3H)-labelled plasmid DNA (0.1 ml) were layered onto 5.0 ml 5-20% linear alkaline sucrose gradients (0.5 M NaCl, 0.3 M NaOH, 0.2 M EDTA, pH 8.0) in cellulose nitrate centrifuge tubes (Beckman). The gradients were centrifuged for 30 min at 35,000 rpm (Beckman L-5-65 ultracentrifuge, SW50.1 rotor). Approximately 35 fractions (7 drops each) were collected from the bottom of the tube directly into scintillation vials containing 1.0 ml of 1 M phosphate buffer and 10 ml Beckman Redi-Solv-scintillation cocktail was added to each vial. Each sample was counted for radioactivity as described above.

b. Neutral Sucrose Gradients

For analytical purposes, samples of (^3H)-DNA were overlaid onto 5 ml linear neutral sucrose gradients (0.55 M NaCl, 0.05 M Tris-HCl, 0.005 M EDTA, pH 8.0). The gradients were centrifuged for 120 min at 35,000 rpm (Beckman L-5-65 ultracentrifuge, SW50.1 rotor), fractionated, and radioactivity determined as described above, except for the exclusion of the 1.0 ml of phosphate buffer.

Preparative linear 5-20% neutral sucrose gradients were used to separate and purify plasmid DNA. Samples of DNA (1.0 ml) were layered

onto 11 ml 5-20% neutral sucrose gradients in 12 ml polyallomar centrifuge tubes and centrifuged in a SP269 swinging bucket rotor at 30,000 rpm, 140 min in an IEC-B60 centrifuge. A bottom dripper was used to divide the gradients into 18 drop fractions. A 5 μ l sample of each fraction was spotted onto filter strips, precipitated with TCA, rinsed in 95% ethanol, dried and counted as previously described. Fractions containing plasmid DNA were pooled, dialyzed against TES buffer and recentrifuged in 12 ml neutral sucrose gradients.

13. Quantitation of DNA

Samples of DNA were quantitated by determining their absorbancy at 260 nm. Plasmid DNA samples were diluted 1:20 in 0.1X SSC and placed in quartz spectrometer tubes. The absorbance at 260 nm, determined using a Gilford UV spectrometer, was equal to the concentration of the undiluted DNA in mg/ml.

14. Electron Microscopic Analysis of Plasmid DNA

A modification of the Kleinschmidt technique (51) was used to prepare the DNA for examination in the electron microscope (EM). To prepare the 400 mesh EM grids, they were first placed on a stainless steel screen set in a large funnel containing double distilled deionized water (ddH_2O). A drop of parlodian dissolved in ethanol was allowed to spread over the water surface and was then lowered onto the screen by slowly releasing the water through the bottom of the funnel. The parlodian coated grids were incubated at 60 C for 15 min and cooled. Prior to each use, a fresh stock of spreading solution

containing 5 μ l of 1.0 M ammonium acetate, 10 μ l cytochrome C (10 mg/ml in ddH₂O) and 40 μ l DNA sample (approximately 1 μ g) in 0.01 M Tris-HCl pH 8.0 was prepared. The stock staining solution, which contained 0.05 M HCl and 0.05 M uranylacetate in 95% ethanol, was made fresh each week.

The DNA was spread using an acid-cleaned microscope slide soaked in hypophase (0.25 M ammonium acetate). The slide was partially submerged in a petri dish containing hypophase and allowed to dry. A 50 μ l drop of spreading solution containing the DNA was gently deposited onto the slide one-eighth inch above the meniscus and allowed to spread over the hypophase for 60 sec. By touching the film side of the grid to the surface of the hypophase near the slide, a drop containing DNA was collected on the grid. The grid was immediately submerged in the staining solution for 30 sec, washed in isopentane for 10 sec, and air dried.

Contrast was enhanced by rotary shadowing using 3 cm of 80:20 platinum:palladium 8 mil wire wrapped around a tungsten wire of 0.025 cm diameter. The grids were viewed through the RCA electron microscope at Hines V. A. Hospital.

The images of the electron-micrograph negatives were projected through a photographic enlarger onto newsprint and traces. Projection enlargement was determined by projecting a centimeter rule at the same focal length. The contour length of each molecule was determined by at least two measurements with a map ruler. A standard EM grid (#1002 having 2159 intervals per mm) was used to determine the magnification

of the electron microscope. A typical grid photograph measured 27 mm per 8 intervals, thus the magnification of this grid was calculated to be 7287. The measured contour length of the molecule divided by the total magnification (7287 X the magnification of the projected image) equaled the true length of the plasmid molecule in microns. Molecular mass in daltons was calculated by multiplying the length of the molecule by the constant 2.07 (59).

CHAPTER III

RESULTS

1. Resistance Phenotype

Proteus mirabilis 2138 is a clinical isolate from the urine of a patient at Hines Veterans Administration Hospital at Hines, Illinois. The resistance to various antibacterial agents of this strain was determined by the Kirby-Bauer disk diffusion technique as described in Materials and Methods. The strain was found to be resistant to ampicillin, carbenicillin, gentamicin, tobramycin, neomycin, kanamycin, streptomycin, tetracycline, colistin, cephalothin and nitrofurantoin, and sensitive to naladixic acid, rifampicin, and chloramphenicol.

2. Transmissibility

To determine if resistance to beta-lactam and aminoglycoside antibiotics in strain 2138 resided on a conjugable plasmid, a mating using Escherichia coli J53-1 as recipient was performed. To select against the Proteus donor, the genetically characterized strain J53 was made resistant to rifampicin (200 µg/ml) by selection on medium containing increasing concentrations of that drug as described in Materials and Methods. This strain, J53-1, was used in subsequent experiments. Minimal medium plates containing methionine, proline, rifampicin (200 µg/ml), and either carbenicillin (100 µg/ml) or gentamicin (50 µg/ml) were used to select transconjugates. Use of the standard overnight broth conjugation procedure described in the Methods section failed to demonstrate transfer of antibiotics resistance to the recipient.

To increase cell to cell contact and hence the probability of conjugation, the donor and recipient cells were concentrated onto Millipore filters as described (see Materials and Methods). Using this method transfer of antibiotic resistance traits was achieved. The frequency of conjugation on filters, calculated as the number of transconjugates per input donor cells, averaged 1.4×10^{-2} with a range of 1.3 to 1.5. There was no significant difference in frequency of transfer between cells selected on carbenicillin-containing plates and those selected on gentamicin-containing plates.

Ten strain J53-1 transconjugate clones, numbered J53-1(P1)-J53-1(P10), were checked for purity and found to be auxotrophic for methionine and proline. To determine if more than one resistant trait had been transferred, antibiotic disk diffusion tests were performed on five transconjugate clones from each of the two selective media employed. The results, shown in Table III, confirm that multiple resistance had been transferred to all clones tested. Resistance to Ap, Cb, Gm, Tm, Km, Sm, and Cl was transferred to both groups of transconjugates. However, in no case was resistance to Nm, Tc or Cs ever observed to transfer. Given the cross-reactivity of most aminoglycoside inactivating enzymes, it is interesting to note that resistance to kanamycin was transferred, but not to that of the closely related antibiotic, neomycin.

To achieve a more precise measure of the level of resistance to these antibiotics, the minimal bactericidal concentrations (MBC) were determined as described in Materials and Methods. The MBCs of am-

Table III.
Antibiotic resistance patterns^a of donor, recipient
and transconjugate strains.

Strain	Antibiotic ^b	No. of isolates
<u>P. mirabilis</u> 2138	Ap Cb Gm Tm Km Sm Cl Tc Cs Nm	1
<u>E. coli</u> J53-1	Rm	1
<u>E. coli</u> J53-1(P1)	Ap Cb Gm Tm Km Sm Cl Rm	10/10

^a Determined by the disk diffusion technique (69)

^b Ap - ampicillin, Cb - carbenicillin, Gm - gentamicin, Tm - tobramycin, Nm - neomycin, Km - kanamycin, Sm - streptomycin, Tc - tetracycline, Cs - colistin, Cl - cephalothin, Rm - rifampicin.

picillin, gentamicin, tobramycin, neomycin, kanamycin and streptomycin were determined for strains 2138, J53-1 and the 10 transconjugates. The results, shown in Table IV, reveal high levels of resistance to Ap, Cb, Gm, Tm and Sm for both the donor strain 2138 and the 10 transconjugate clones. Values were very low for the recipient strain J53-1. Variations of one tube dilution were seen in the MBC values of the transconjugates. It is uncertain why the MBC of Sm for 2138 and the transconjugate strains differ. It is possible that the donor has inherent resistance properties which are not transferred. A difference of one tube dilution is not thought to be significant (37). Resistance to high concentrations of antibiotics is indicative of R plasmid-mediated resistance (76). Again the lack of transfer of neomycin resistance was clearly evident, despite the high level of resistance in the donor.

3. Isolation of Extrachromosomal DNA

The fact that resistance to high concentrations of antibiotics could be transferred to a sensitive strain, indicated that an R plasmid was involved. Definitive proof of the existence of plasmids can be obtained through isolation of the plasmid DNA by ultracentrifugation (91). To determine if drug resistance was conferred by an R plasmid, the Proteus donor, J53-1 recipient and J53-1(P1) transconjugate were analyzed for plasmid DNA using the Helinski method for partial purification of plasmid DNA followed by cesium chloride-ethidium bromide density centrifugation (see Materials and Methods). The gradients were centrifuged for 72 h at 32,000 rpm as described. Viewed under UV illumination, satellite DNA bands were visible in the gradients from

Table IV.

Minimal Bactericidal Concentrations^a for donor, recipient
and transconjugate strains.

Strain	MBC ($\mu\text{g/ml}$) ^b						
	Ap	Cb	Gm	Tm	Km	Sm	Nm
<u>P. mirabilis</u> 2138	>2000	>2000	500	500	>1000	>2500	>2000
<u>E. coli</u> J53-1	<8	<8	2	<8	<8	20	<8
Transconjugates ^c							
J53-1(P1)	>2000	>2000	500	500	1000	625	<8
J53-1(P2)	>2000	>2000	250	250	1000	625	<8
J53-1(P3)	>2000	>2000	250	500	500	313	<8
J53-1(P4)	>2000	>2000	250	250	1000	313	<8
J53-1(P5)	>2000	>2000	250	250	500	313	<8
J53-1(P6)	>2000	>2000	250	250	1000	313	<8
J53-1(P7)	>2000	>2000	250	250	500	313	<8
J53-1(P8)	>2000	>2000	250	500	500	313	<8
J53-1(P9)	>2000	>2000	250	500	1000	313	<8
J53-1(P10)	>2000	>2000	250	500	1000	313	<8

^a Determined by tube dilution technique.

^b Abbreviations are as noted in Table III.

^c J53-1(P1) - J53-1(P5) selected on medium containing 50 $\mu\text{g/ml}$ Gm,
J53-1(P6) - J53-1(P10) selected on medium containing 100 $\mu\text{g/ml}$
Cb as described in the Results section.

the donor and transconjugate, but not in the recipient (data not shown).

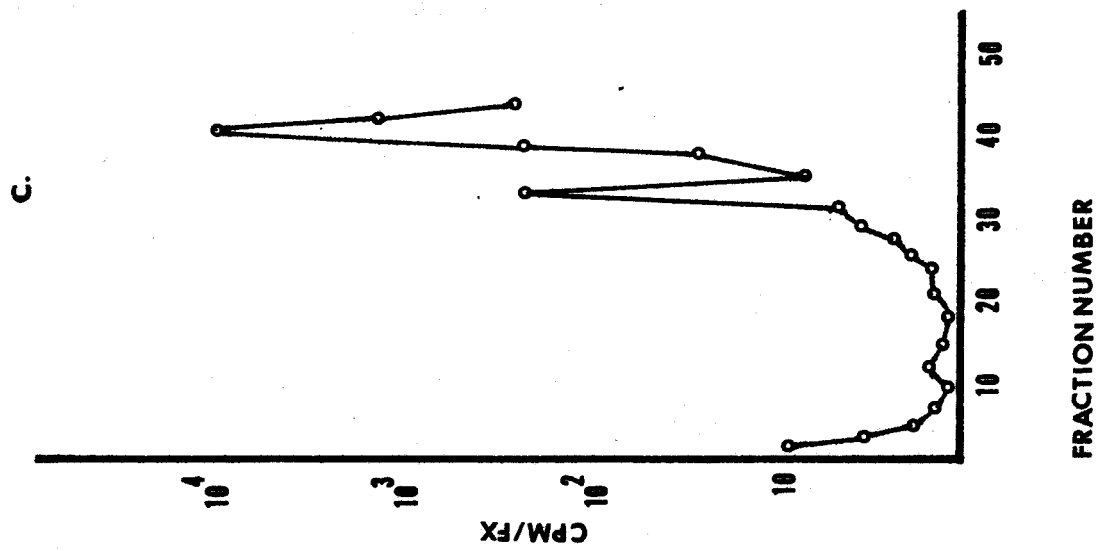
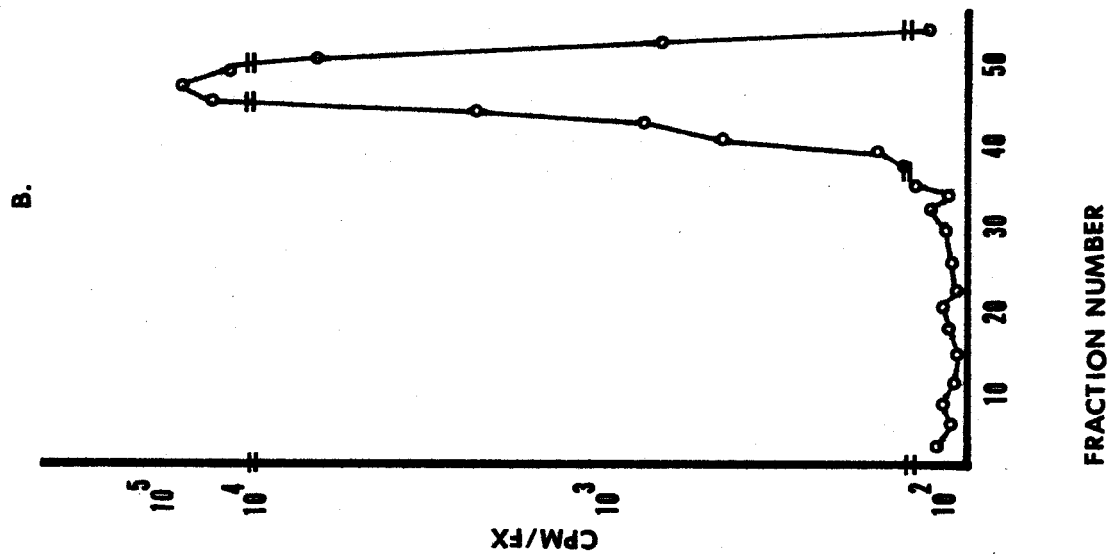
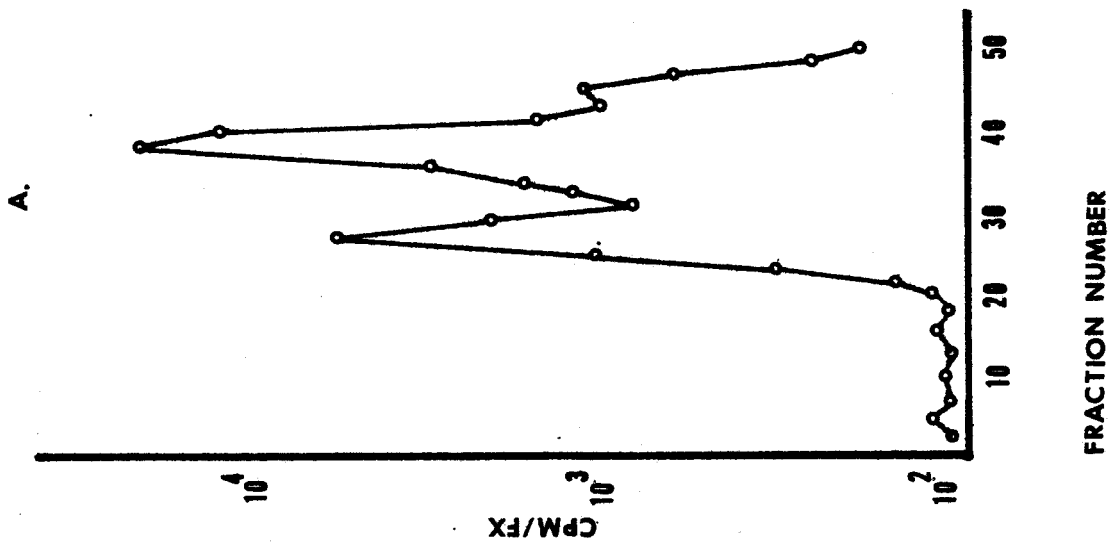
To isolate R plasmid DNA, cells were grown in the presence of (^3H)-thymidine as described in the Methods section, lysed by the Helinski method and the partially purified radiolabelled DNA was centrifuged to equilibrium in CsCl-EtBr gradients. The gradients were fractionated from the bottom and 0.01 ml samples of each fraction were spotted on filter strips. Following TCA precipitation, radioactivity was determined as described in Materials and Methods. Profiles of the gradients, shown in Figure 1, demonstrate the presence of dense satellite DNA in strains 2138 and J53-1(P1), where none could be seen in strain J53-1. The presence of the two peaks in the CsCl-EtBr gradient profile confirmed the visual data described above. The concomitant acquisition of antibiotic resistance and extrachromosomal DNA provided evidence for the existence of a conjugable R plasmid in P. mirabilis 2138.

4. Molecular Characterization of the Plasmid

a. Determination of Plasmid Size

The most accurate molecular mass determinations of plasmid molecules have come from measurement of the contour length of the molecules taken from electron micrographs (43). Preparative (500 ml) cultures of strains 2138 and J53-1(P1) were lysed according to the Helinski method. To enhance the yield and concentrate the DNA, the lysates were precipitated with PEG as described in Materials and Methods, and centrifuged to equilibrium in CsCl-EtBr gradients. The plasmid bands were extracted from the gradients and purified by a

Figure 1. Cesium chloride-ethidium bromide centrifugation profiles of DNA isolated from donor recipient and transconjugate strains. Helinski cleared lysates of *P. mirabilis* 2138, *E. coli* J53-1 and *E. coli* J53-1(P1) were centrifuged to equilibrium at 20 C, using a fixed angle 40 rotor in a Beckman L5-65 ultracentrifuge for 60 h at 35,000 rpm. The gradients were fractionated from the bottom and radioactivity determined as described in Materials and Methods. (A) *P. mirabilis* 2138. (B) *E. coli* J53-1 (C) *E. coli* J53-1(P1).



second centrifugation in CsCl-EtBr. The purified plasmid DNA was removed and prepared for electron microscopy using the method of Kleinschmidt as described in the Materials and Methods section.

Typical electronmicrographs are shown in Figure 2. Contour length measurements revealed the presence of at least two size classes of molecules in strain 2138 and only one in strain J53-1(P1). The molecular mass distributions of the plasmids are shown in Figure 3. Plasmids from strain 2138 were found to be 4.96 ± 0.05 and 25.82 ± 2.82 megadaltons (Figure 3A). The single size class found in strain J53-1(P1) measured 89.76 ± 2.23 megadaltons (Figure 3B).

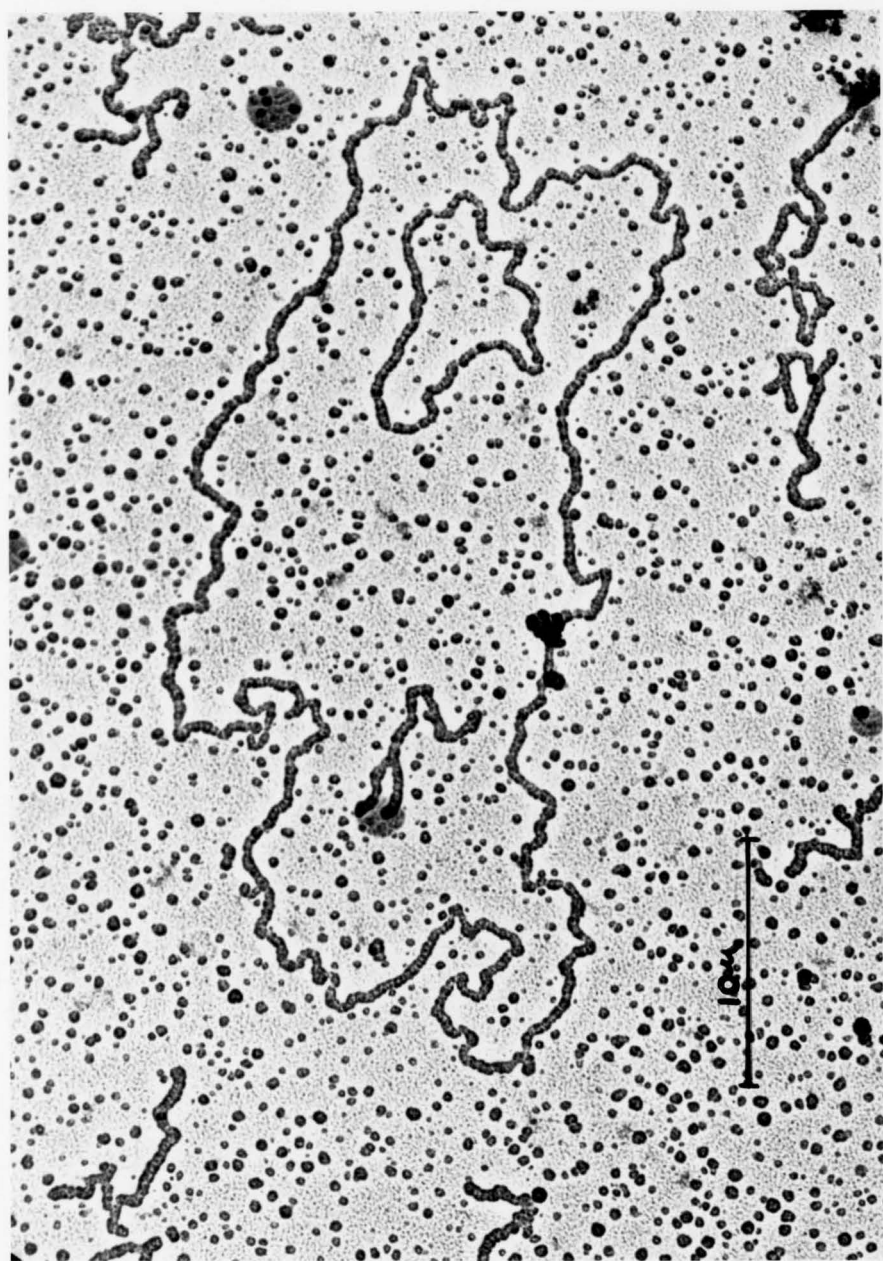
Existence of dissociated R plasmids in Proteus species has been well documented (11, 36, 93, see Introduction). If this were the case in strain 2138, the large plasmid found in J53-1(P1) could be a trimer of the 27 megadalton plasmid found in strain 2138. Further elucidation of the molecular nature of these plasmids was needed to determine the relationship between them.

b. Neutral Sucrose Gradients

To determine whether the small and large Proteus plasmids represented r-determinants and RTF replicons respectively, it would be necessary to isolate them individually and use the DNA to transform sensitive strains. Neutral sucrose gradients provide an effective means of separating plasmids on the basis of their molecular mass, and were used to separate the plasmids present in strains 2138 and J53-1(P1).

Large cultures (500 ml) of strains 2138 and J53-1(P1) were grown as described previously. Additionally, 30 ml cultures of each strain

Figure 2. Electronmicrographs of plasmids isolated from donor and transconjugate strains. The Helinski lysis technique followed by dye-bouyant density centrifugation was used to isolate DNA from P. mirabilis 2138 and E. coli J53-1(P1). The isolated DNA was prepared for electron microscopy by the Kleinschmidt method (see Materials and Methods section). (A) P. mirabilis 2138 (B) E. coli J53-1(P1)



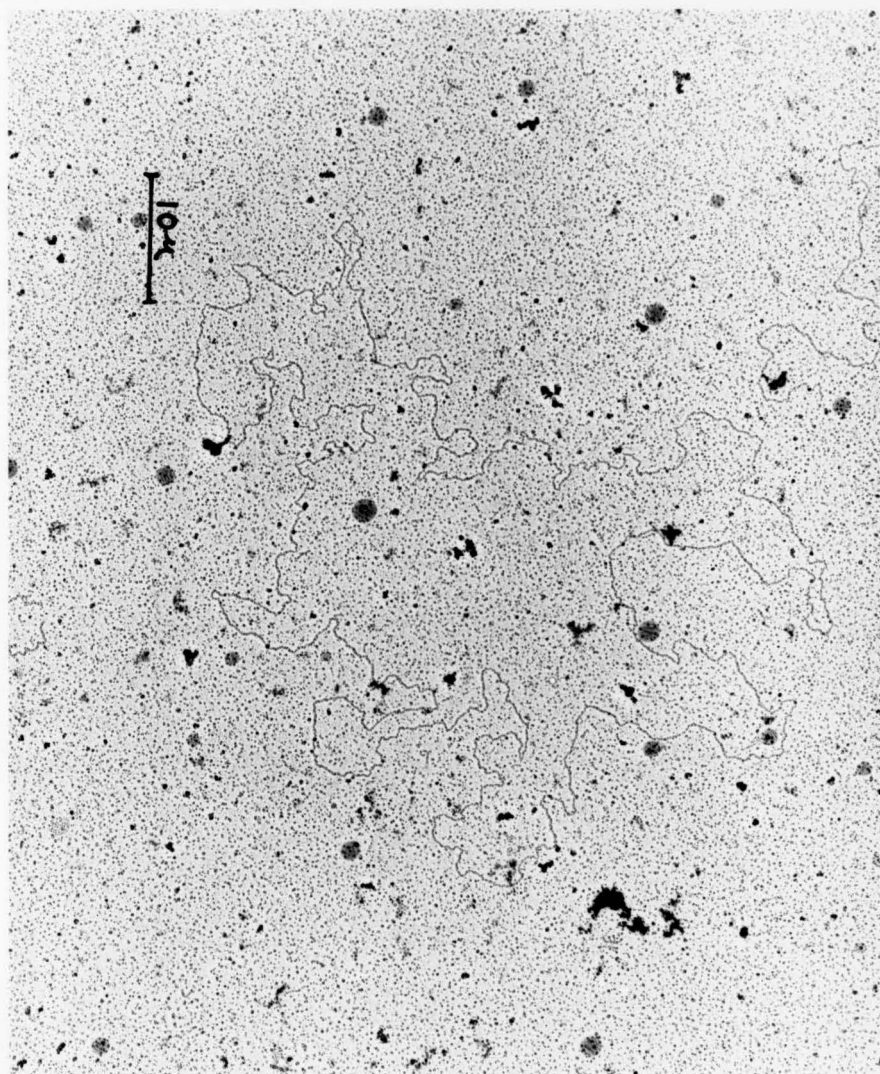
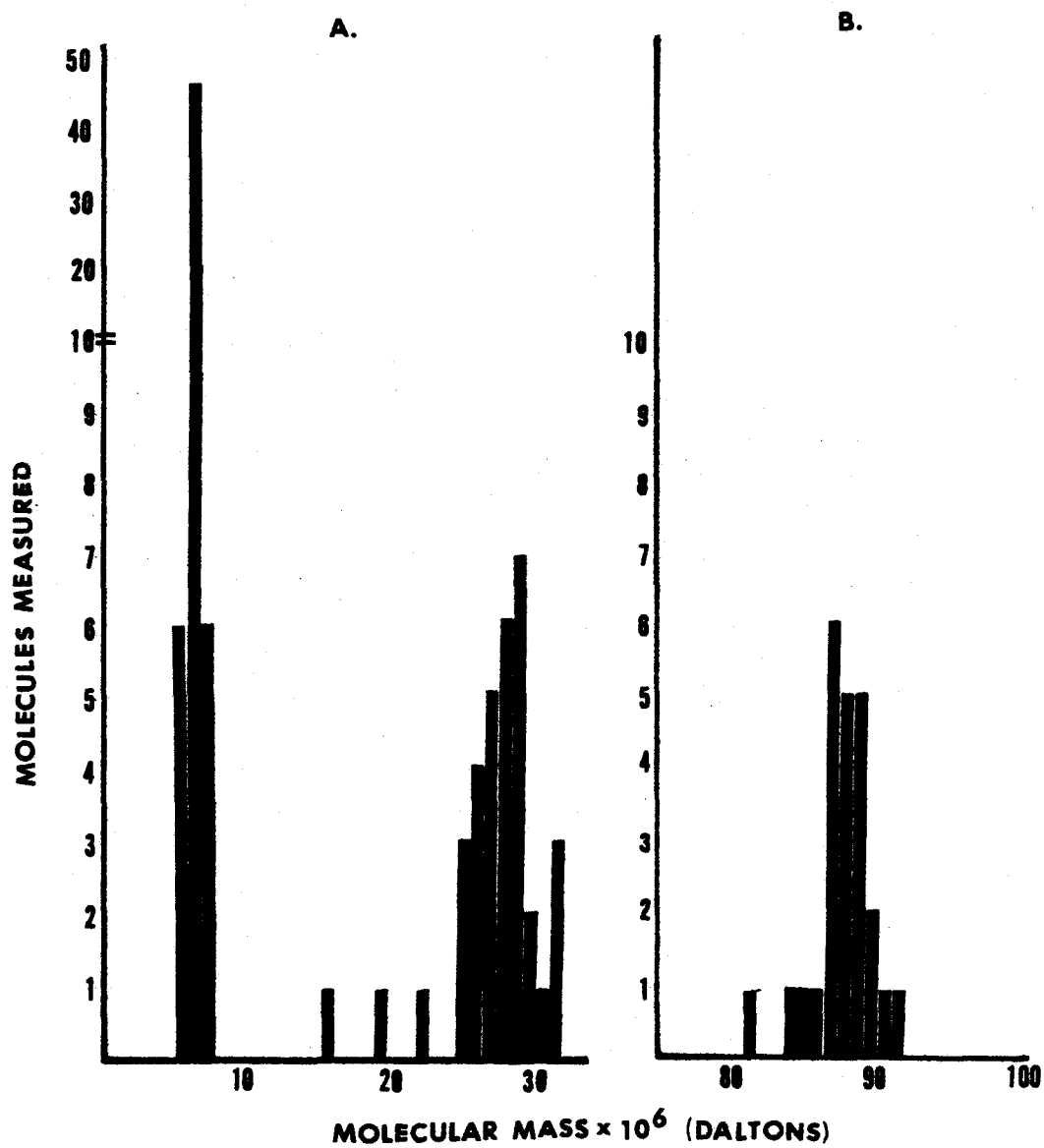


Figure 3. Molecular mass distributions of plasmid molecules isolated from P. mirabilis 2138 and E. coli J53-1(P1). Plasmid DNA was isolated using the Helinski cleared lysate technique, purified by equilibrium gradient centrifugation in cesium chloride-ethidium bromide and prepared for electron microscopy using the method of Kleinschmidt (see Materials and Methods). Contour length measurements of electron micrographs were converted to molecular mass using the equation: $\text{Mass} = 2.07 \times \mu\text{m}$. (A) P. mirabilis 2138 plasmid DNA. (B) E. coli J53-1(P1) plasmids DNA.

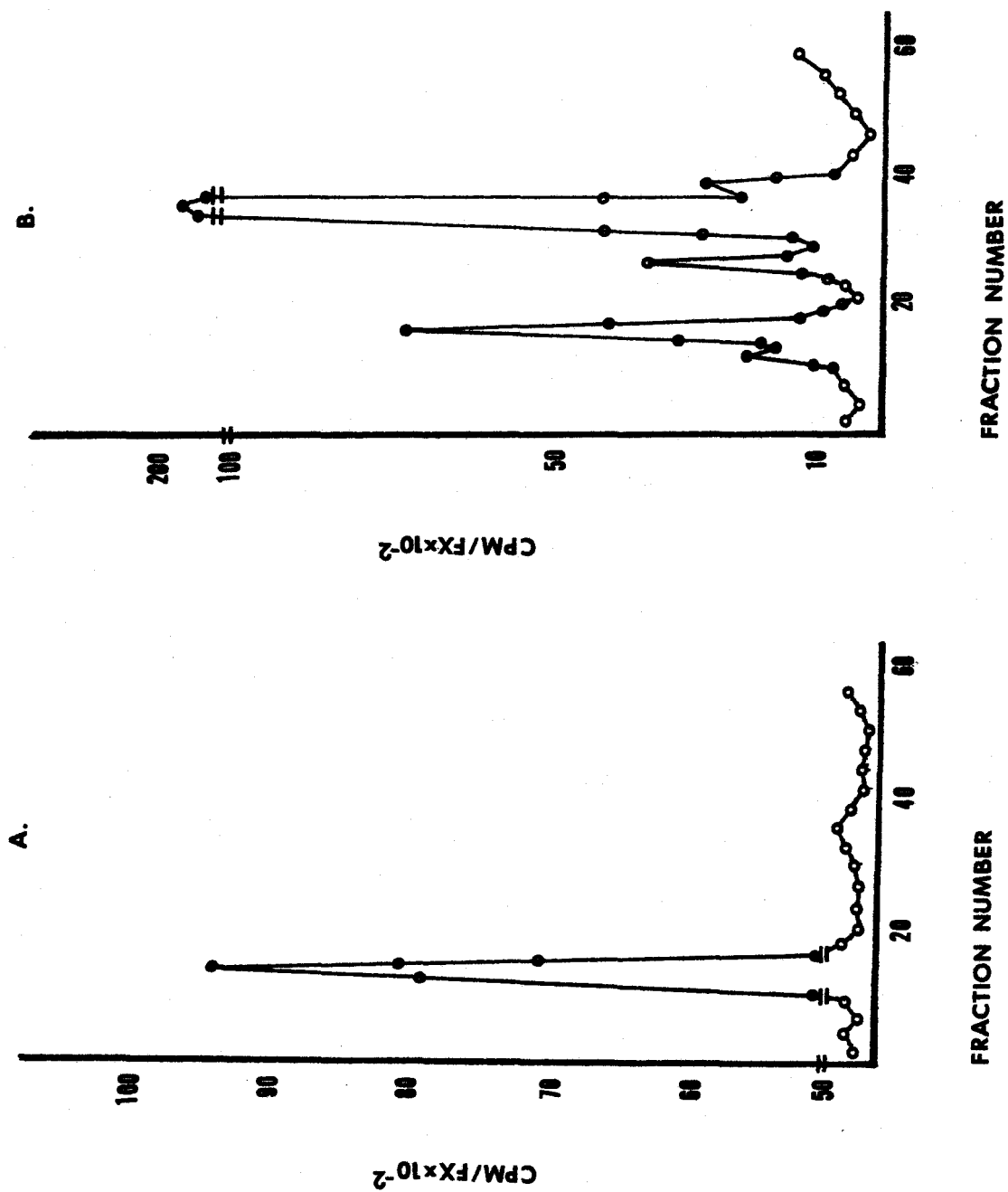


were grown in the presence of (^3H)-thymidine as described in Materials and Methods. The cells from both cultures were combined and lysed by the Helinski method followed by PEG precipitation. A 0.1 ml sample of the isolated plasmid DNA, twice purified in CsCl-EtBr gradients, was overlayed onto a 5 ml analytical 5-20% linear neutral sucrose gradient and centrifuged at 35,000 rpm for 150 min (see Materials and Methods section). The sucrose gradient profiles are shown in Figure 4. As expected the presence of one fast sedimenting plasmid was seen in the profile of plasmid DNA from strain J53-1(P1) (Figure 4A). However, the profile of plasmid DNA from strain 2138 (Figure 4B) was not as clear. At least three peaks could be distinguished in the gradient, but the data was irreproducible. The existenance of several diffuse peaks were seen in later gradients.

c. Alkaline Sucrose Gradients

Initially it was thought that the poor resolution of the Proteus gradient was due to breakage during the extraction and isolation, resulting in nicked open circular or broken linear fragments of the plasmid DNA. These species would sediment at different rates and could account for multiple, nonreproducible peaks in neutral sucrose gradients. Alkaline sucrose gradient sedimentation causes denaturation of nicked and linear forms of DNA. Since supercoiled DNA will sediment 3.8 times faster than the denatured forms in alkaline sucrose gradients (pH 12.5) (100), the number and size of resident plasmid species within the Proteus strain could be determined. Radiolabelled plasmid DNA from strains 2138 and J53-1(P1) was isolated

Figure 4. Neutral sucrose gradient profiles of plasmid DNA from strains J53-1(P1) and 2138. The DNA samples were isolated by the Helinski method, twice purified in cesium chloride-ethidium bromide gradients and overlayed on a 5-20% linear neutral sucrose gradient in a SW50.1 rotor. The gradients were centrifuged at 20 C, 35,000 rpm for 2.5 h as described in Materials and Methods. (A) Strain J53-1(P1). (B) Strain 2138.



using the Currier method and subjected to alkaline sucrose gradient sedimentation as described in Materials and Methods. The gradient profiles from strain 2138, shown in Figure 5, revealed the presence of the two plasmids seen in electronmicrographs as well as the presence of a third, fast sedimenting DNA species. This suggested that a third plasmid was present in strain 2138, but had not been isolated in quantity using the Helinski lysis technique.

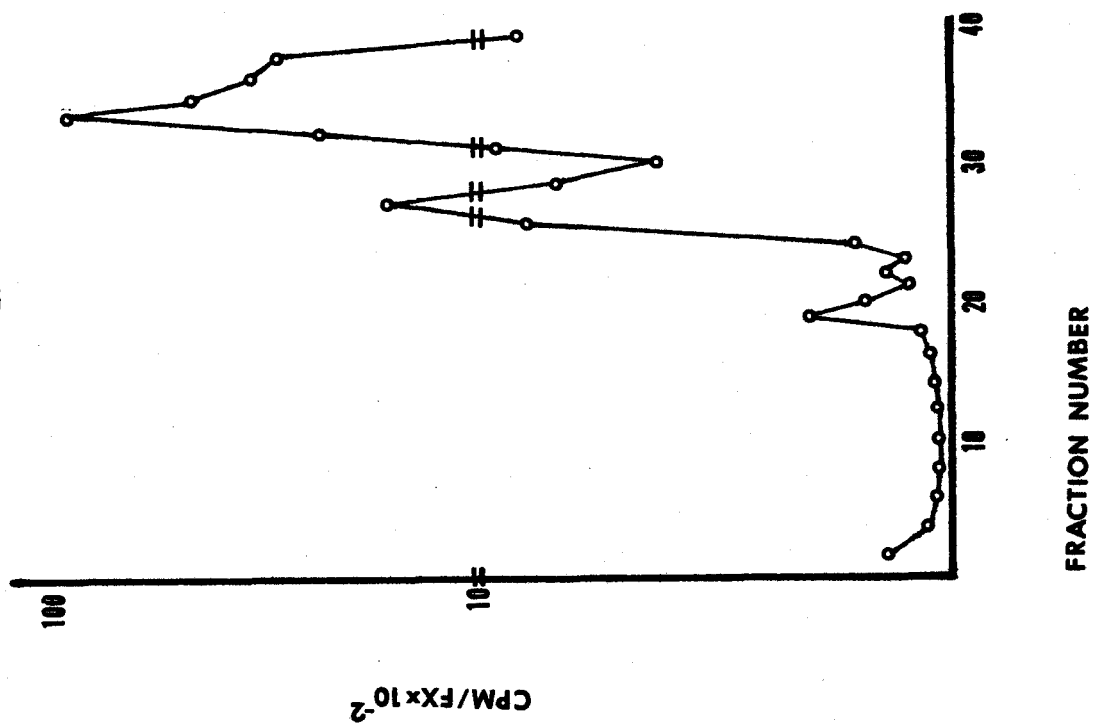
d. Isolation of the Large Plasmid

The Currier plasmid isolation technique was employed for its usefulness in isolating large plasmids having molecular mass in excess of 60 megadaltons (15). Large (500 ml) cell cultures were combined with 30 ml cultures of cells grown in the presence of (^3H)-thymidine, lysed and subjected to the Currier plasmid isolation technique as described in Materials and Methods. Dye-bouyant centrifugation of the DNA revealed the presence of satellite bands from lysates of strains 2138 and J53-1(P1), but not in strain J53-1. This reaffirmed that plasmid transfer had occurred between donor and recipient strains. It was found that a modification of the Currier method (see Materials and Methods) using sodium acetate and ethanol to precipitate the DNA greatly enhanced the yield of plasmid DNA. Figure 6 shows the CaCl_2 -EtBr gradient profiles of strain 2138 and J53-1(P1) using this technique. The plasmid fractions were pooled, rebanded in CsCl -EtBr gradients and prepared for sucrose gradient sedimentation.

The analytical sucrose gradient sedimentation profiles of plasmid DNA from strains 2138 and J53-1(P1) isolated using the modified

Figure 5. Alkaline sucrose gradient profiles of plasmid DNA from strains 2138 and J53-1(P1). The DNA samples were isolated by the Currier method and purified by centrifugation in cesium chloride-ethidium bromide density gradients. The isolated plasmid DNA samples were layered on 5-20% alkaline (pH 12.0) sucrose gradients and centrifuged in a SW50.1 rotor, 35,000 rpm for 0.5 h. Fractions were collected from the bottom and analyzed as described in Materials and Methods. (A) Strain J53-1(P1). (B) Strain 2138.

B.



A.

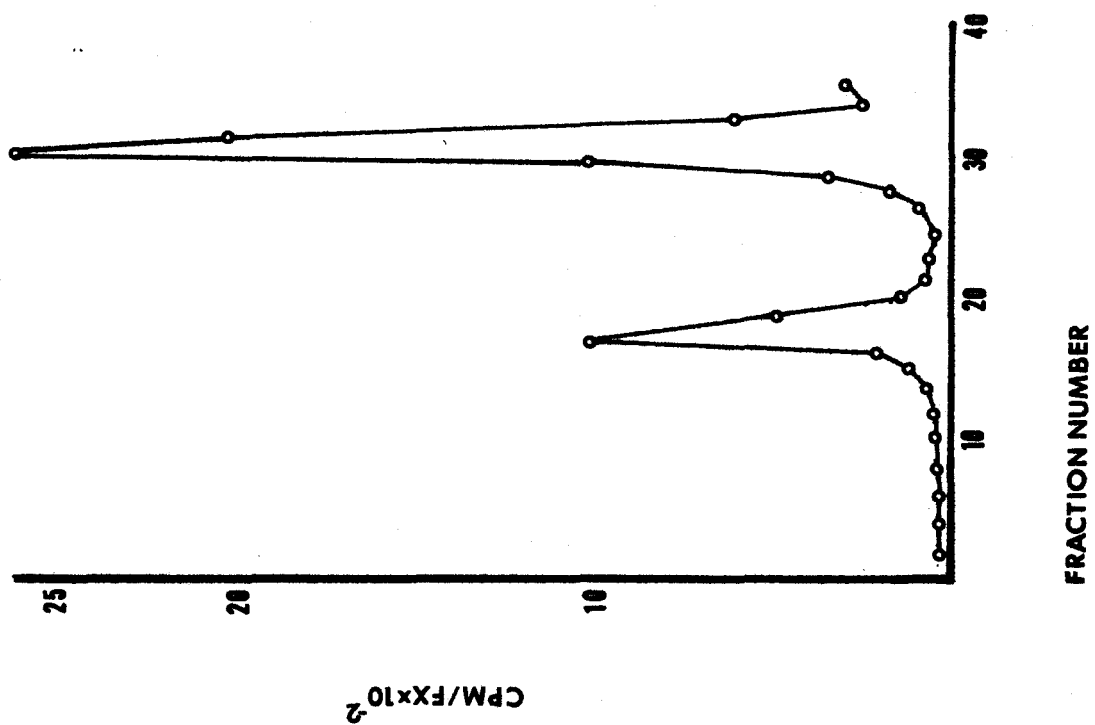
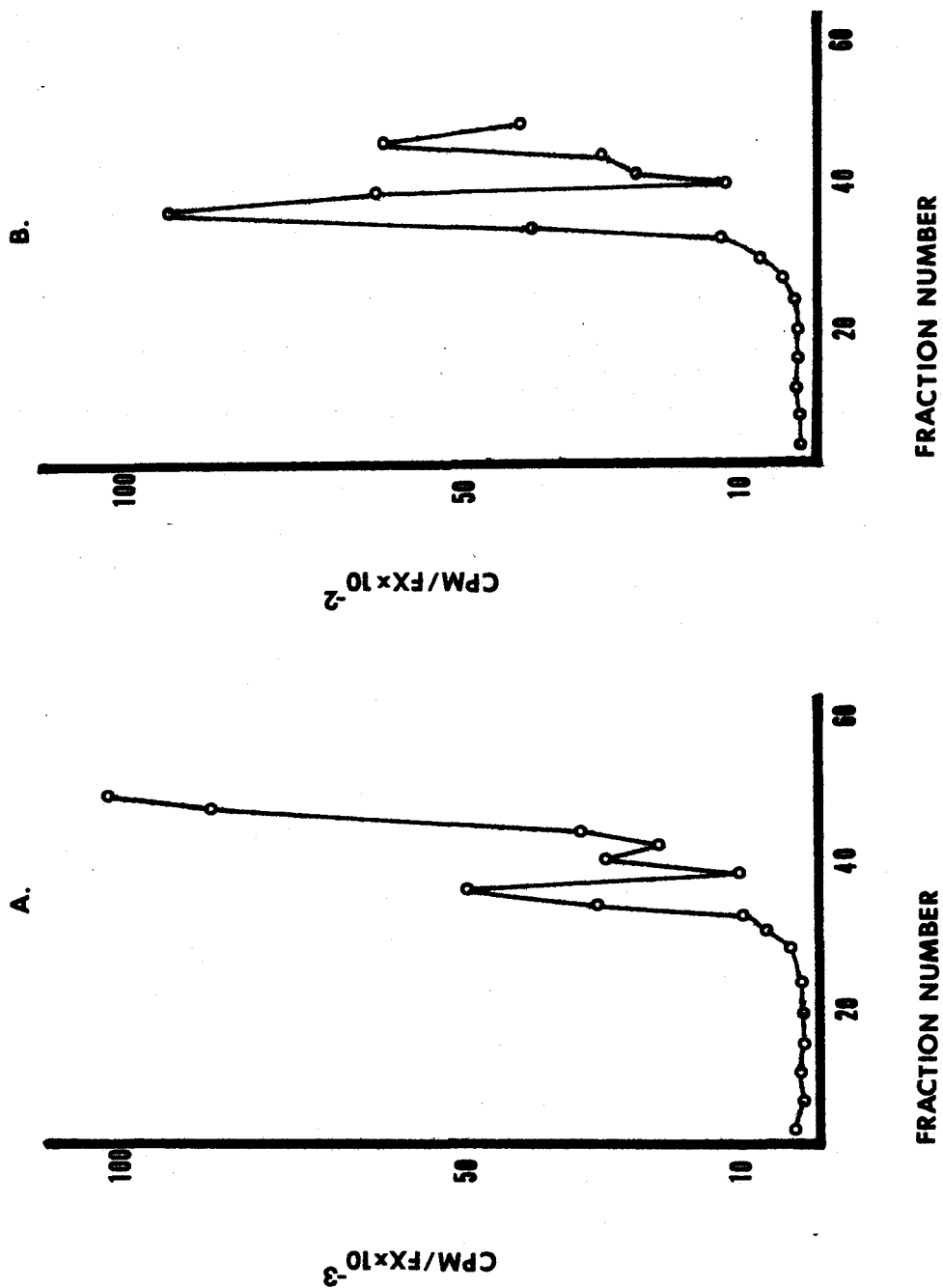


Figure 6. Cesium chloride-ethidium bromide profiles of purified plasmid DNA from strains 2138 and J53-1(P1). Plasmid DNA was isolated using the Currier technique and purified by cesium chloride-ethidium bromide density centrifugation. The CsCl-EtBr gradients were centrifuged, fractionated and analyzed as described for Figure 1. (A) Strain J53-1(P1). (B) Strain 2138.



Currier technique are shown in Figure 7. One peak was seen in the strain J53-1(P1) gradient (Figure 7A), while three, clearly distinguishable peaks were seen in the strain 2138 plasmid DNA gradient (Figure 7B). The slowest sedimentating peak was labelled peak I, the middle as peak II, and the fastest as peak III.

e. Separation of Proteus plasmids

Preparative neutral sucrose gradients were employed to isolate and separate the three Proteus plasmids in quantity. The cells were grown as before and subjected to the modified Currier plasmid isolation technique. Plasmid DNA was centrifuged in CsCl-EtBr gradients twice and prepared for neutral sucrose gradient sedimentation. A 1.0 sample of purified DNA was overlayed on a 12 ml linear 5-20% neutral sucrose gradient, and centrifuged at 30,000 rpm in a SP269 swinging bucket rotor for 140 min (see Materials and Methods). The gradients were fractionated by bottom puncture and radioactivity in each fraction determined as described. Fractions of each plasmid peak were pooled and centrifuged separately in a second sucrose gradient. The three Proteus plasmids were collected as described in Materials and Methods and used for further study.

f. Determination of Plasmid Size

Electron micrographs of the largest (peak III) Proteus plasmid isolated by neutral sucrose gradient sedimentation, were taken and used to size this plasmid species. A typical picture is shown in Figure 8. The molecular mass, calculated from the contour length

Figure 7. Neutral sucrose gradient profiles of plasmid DNA isolated from strains 2138 and J53-1(P1). Plasmid DNA isolated by the Currier method was purified by two centrifugations in cesium chloride-ethidium bromide gradients, collected and prepared for neutral sucrose gradient sedimentation as described in the Materials and Methods section. The 12 ml sucrose gradients (5-20%) were centrifuged in a SP269 rotor at 30,000 rpm for 140 min at 20 C and fractionated by bottom puncture. Radioactivity was determined as described in Materials and Methods. (A) Strain J53-1(P1). (B) Strain 2138.

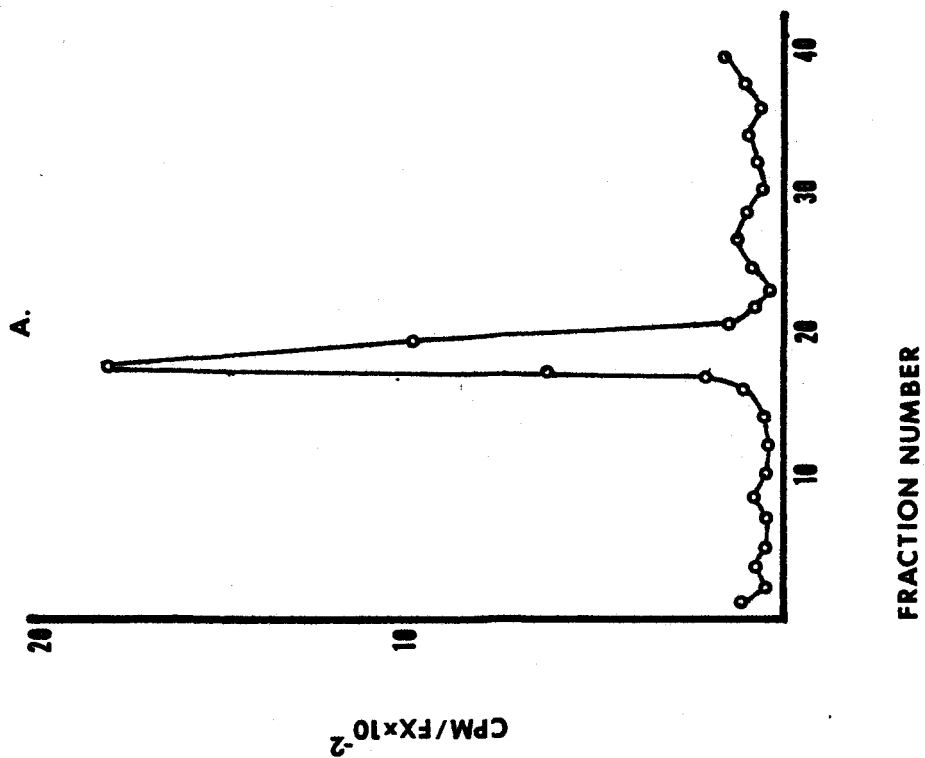
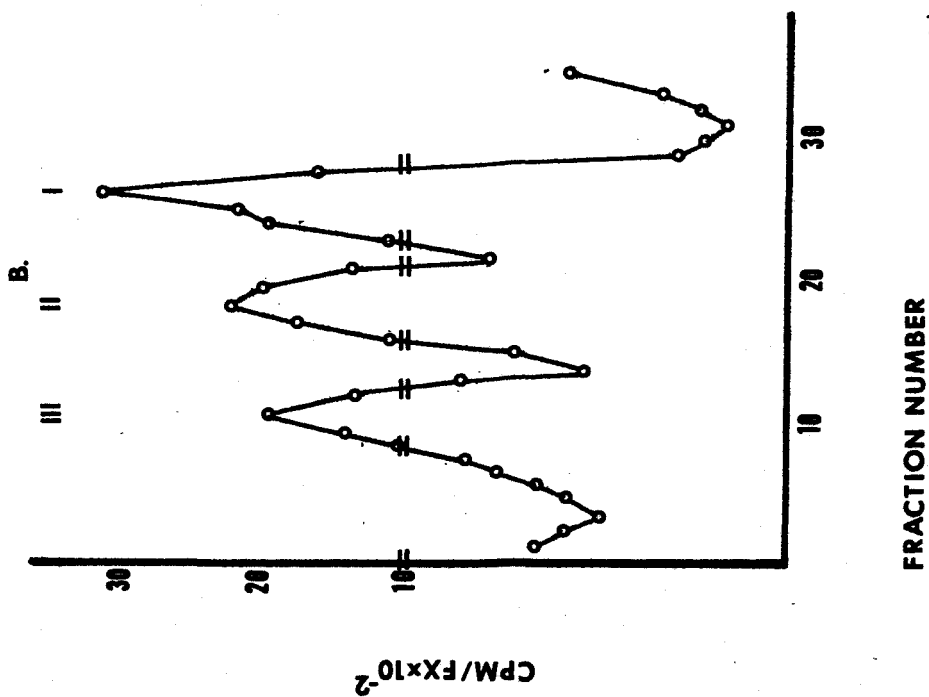
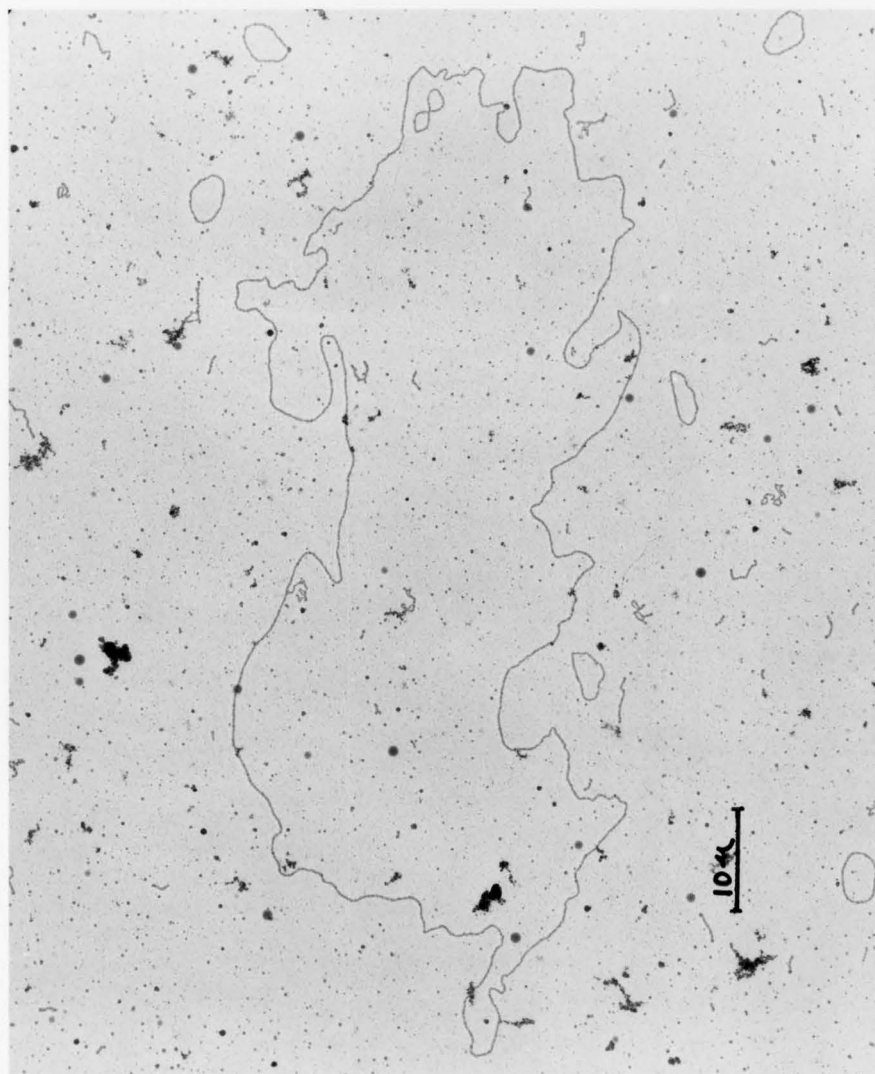
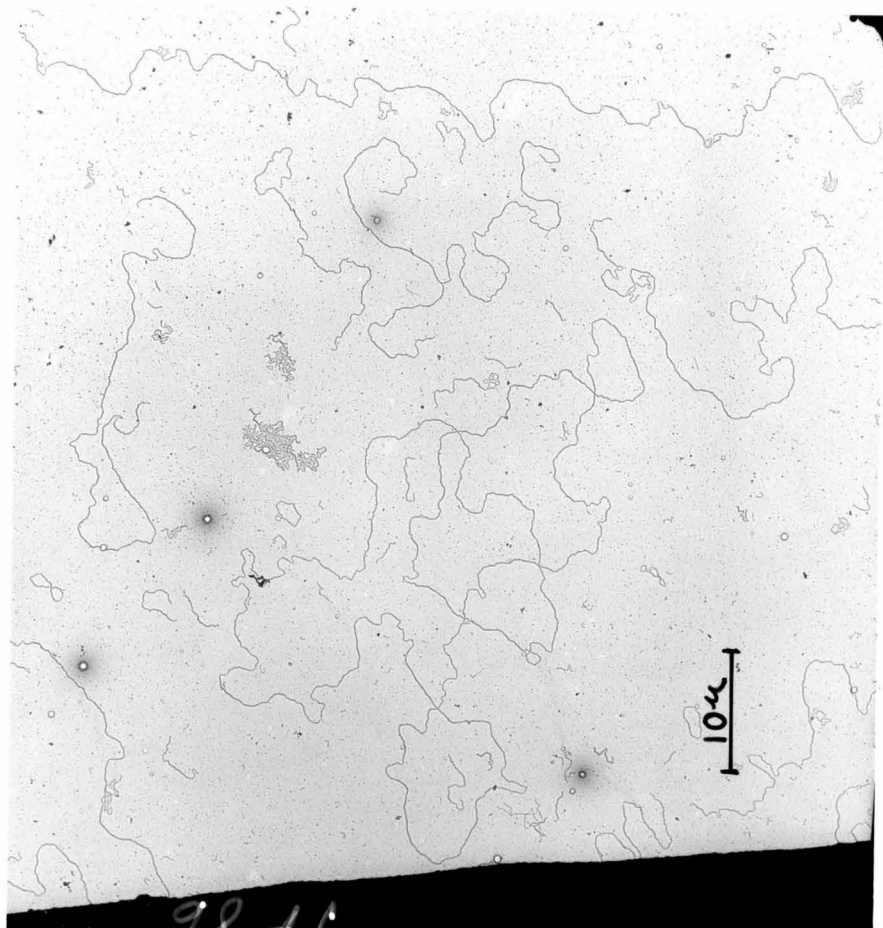


Figure 8. Electron micrographs of P. mirabilis 2138 peak III plasmid DNA. Proteus plasmid DNA was isolated using the Currier technique and purified by cesium chloride-ethidium bromide centrifugation. The Proteus plasmids were separated according to mass centrifugation in neutral sucrose gradients as described in Materials and Methods. Peak III plasmid DNA was prepared for viewing under the electron microscope using a modification of the Kleinschmidt method as described in Materials and Methods. A typical molecule is shown here.





measurements, was found to be close to that of the J53-1(P1) plasmid. The distribution of the measured plasmid molecule is shown in Figure 9. The average molecular mass of the peak III plasmid was determined to be 90.33 ± 2.45 megadaltons. Compared to a value of 89.87 ± 2.23 megadaltons obtained for the plasmid isolated from J53-1(P1), it was concluded that these were the same plasmid species.

Electronmicrograph measurements of peak I and peak II plasmid DNA were consistent with previous measurements of strain 2136 plasmid DNA. Peaks I and II plasmid DNA averaged 4.95 and 27.1 megadaltons respectively. It was concluded that these were the same plasmid series seen in Figure 2.

g. Agarose Gel Electrophoresis

Agarose gel electrophoresis has been used to detect and estimate plasmid DNA species ranging in molecular mass from 6.0×10^5 to 95×10^6 daltons (78). Isolated plasmid DNA from strains 2138 and J53-1(P1) were electrophoresed in horizontal agarose gels as described in the Materials and Methods section. The gel, pictured in Figure 10, reveals the presence of three plasmid species in Proteus and one in strain J53-1(P1). Slots A and H contain total P. mirabilis 2138 plasmid DNA and clearly reveal the presence of 3 plasmid species. The plasmid DNA from peak I, seen in slots B and C aligns with the fastest migrating plasmid in slot A. Above the chromosomal band in slot A is the middle sized plasmid which migrates with the peak II plasmid in slot D. The peak II plasmid in slot E is not visible. The slowest migrating plasmid in slot A aligns with the peak III plasmid

Figure 9. Molecular mass distribution of peak III Proteus plasmids. Plasmid DNA isolated from strain 2138 by the Currier method was twice centrifuged in cesium chloride-ethidium bromide gradients and purified in neutral sucrose gradients as described in the Materials and Methods section. The DNA was prepared for electron microscopy by a modification of the Kleinschmidt method. Molecular mass was determined as for Figure 3.

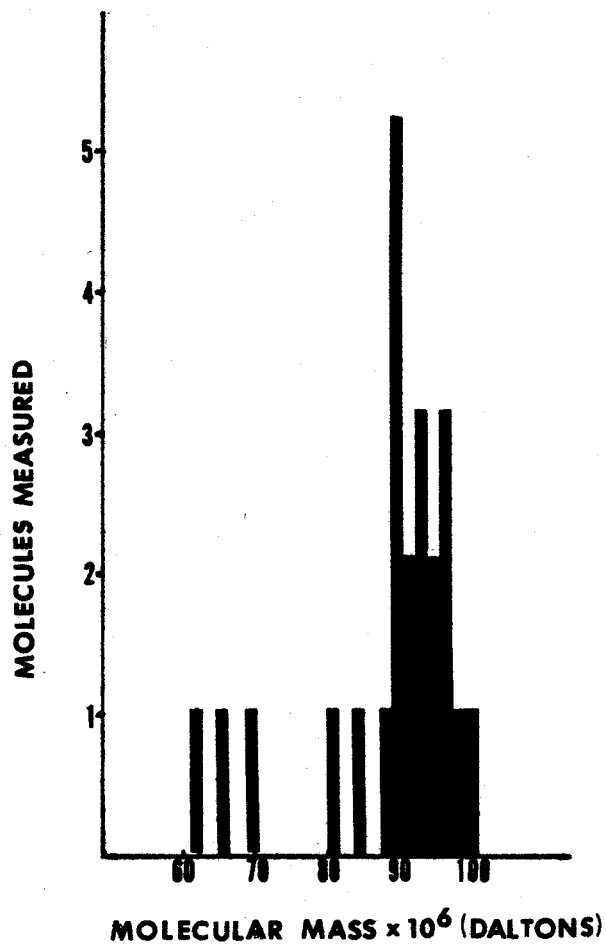


Figure 10. Agarose gel electrophoresis of purified plasmid DNA isolated from strains 2138 and J53-1(P1). Plasmid DNA from strains 2138 and J53-1(P1) was isolated as for Figure 9. The purified plasmid DNA was subjected to electrophoresis in a horizontal 0.7% agarose gel for 4 h at 120 V. The gel was stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) for 15 min. Pictures were taken with a Poloroid MP4 camera using type 57 film under UV illumination. (A) Total strain 2138 plasmid DNA. (B and C) Isolated peak I plasmid DNA. (D and E) Isolated peak II plasmid DNA. (F) Isolated peak III plasmid DNA. (G) Isolated J53-1(P1) plasmid DNA. (H) Total strain 2138 plasmid DNA.

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III

II

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I

H

G

F

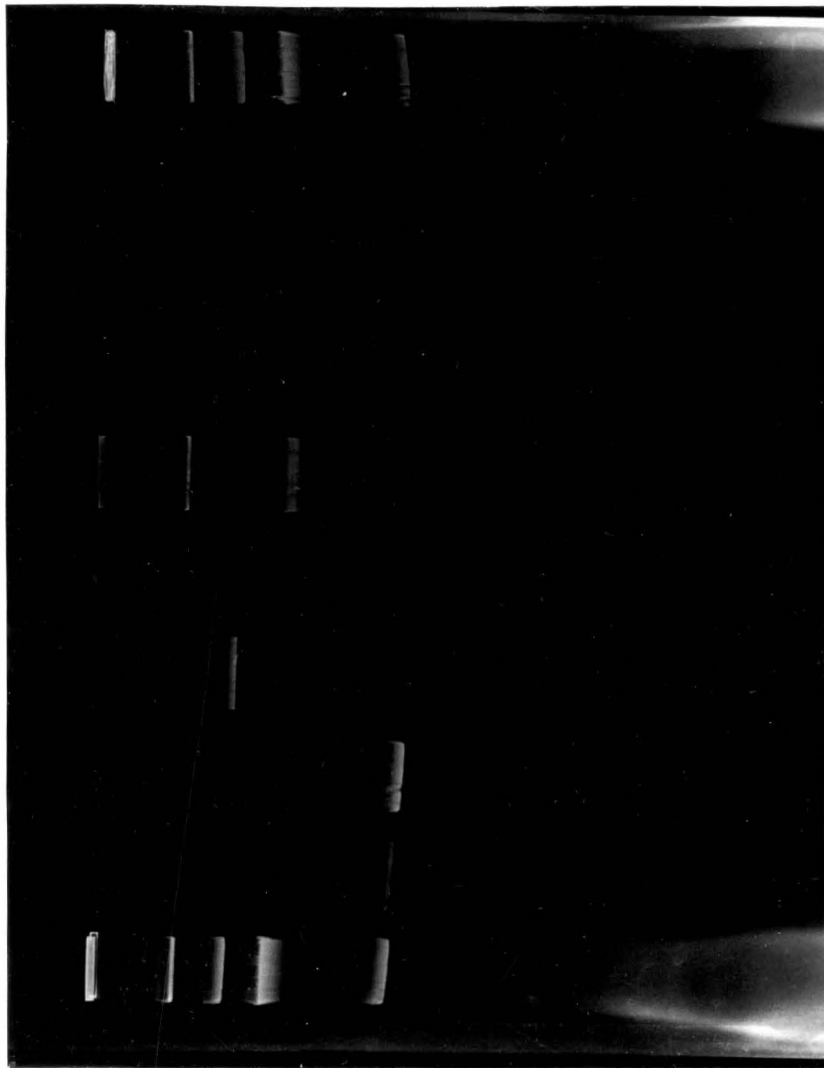
E

D

C

B

A



in slot F and the J53-1(P1) plasmid in slot G. This supports the contour length measurements which showed that the peak III plasmid and the J53-1(P1) plasmid were the same size.

5. Proof that the Peak III Plasmid Confers Aminoglycoside Resistance

a. Transformation Experiment

A transformation experiment was devised to determine if the 90 megadalton plasmid (peak III) was responsible for resistance to the beta-lactam and aminoglycoside antibiotics. Individual plasmid DNA species isolated from strain 2138 by CsCl-EtBr centrifugation of Currier lysates and separated in neutral sucrose gradients were used as the transforming elements. A rifampicin resistant clone of Escherichia coli C, strain C1200-1 (his, met), was selected as recipient because it lacks a restriction system. The transformation method of Lederberg and Cohen was followed as described in the Materials and Methods section, and transformants were selected on plates containing either 25 µg/ml gentamicin or 100 µg/ml carbenicillin.

Only two of the individual plasmid DNA species isolated from Proteus mirabilis 2138 were found to be capable of transforming strain C1200-1 to antibiotic resistance. Both the peak I (4.96 Mdal) and peak III (90.3 Mdal) plasmids were capable of transforming strain C1200-1 to carbenicillin resistance. However, only the latter was capable of transforming strain C1200-1 to gentamicin resistance. No gentamicin or carbenicillin resistant transformants could be isolated

using the peak II (26.8 Mdal) plasmid. The frequency of transformation, calculated as the number of transformants per input donor cell, was determined for each plasmid species. Transformation of Cb resistance occurred at a much higher frequency using the peak I plasmid donor (7.2×10^{-4}) than the peak III plasmid donor (7.3×10^{-8}). Transformation of gentamicin resistance occurred at a frequency of 3.8×10^{-8} . The ability of the smaller plasmid to transform strain C1200-1 at a higher frequency than the large plasmid was thought to be either a reflection of the size difference between the two plasmids, an effect of concentration of donor DNA, or a combination of both factors.

Escherichia coli C1200-1 transformants of each plasmid species were cloned, tested for purity and subjected to antibiotic disk diffusion testing to determine if additional antibiotic resistance traits had been transferred. Ten clones from each selection were tested and the results are shown in Table V. Peak I transformants demonstrated resistance exclusively to the beta-lactam antibiotics. Peak III transformants selected on medium containing either gentamicin or carbenicillin showed identical resistance patterns including resistance to Ap, Tm, Km, Sm and Cl in addition to Gm and Cb.

b. Electrophoresis of Plasmid DNA

To detect the presence of the plasmid DNA in the transformants and to determine if this was the same size species as the donor plasmid, the strain C1200-1 transformants were lysed, and the partially purified plasmid DNA samples were subjected to agarose gel electrophoresis as described in Materials and Methods. The results,

Table V.

Transformation of E. coli to antibiotic resistance using
P. mirabilis plasmid DNA^a.

Strain	Transforming DNA ^b	Selection	Resistance pattern ^c
<u>P. mirabilis</u> 2138	-	-	Ap Cb Gm Tm Km Sm Cs Cl Nm Tc
<u>E. coli</u> C1200-1	-	-	
Transformants			
C1200-1	peak I	Ap	Ap Cb Cl
C1200-1	peak I	Gm	No transformants detected
C1200-1	peak II	Ap	No transformants detected
C1200-1	peak II	Gm	No transformants detected
C1200-1	peak III	Ap	Ap Cb Gm Tm Km Sm Cl
C1200-1	peak III	Gm	Ap Cb Gm Tm Km Sm Cl

^a Determined by disk diffusion technique (69).

^b Transforming DNA as described as in Figure 7.

^c Abbreviations as described in Table III.

pictured in Figure 11, reaffirm that the peak III plasmid was the conjugable R plasmid. The peak I transformants are shown to have only one plasmid which migrates with the 4.95 Mdal plasmid found in strain 2138. The largest Proteus plasmid migrated with that found in the peak III transformants of strain C1200-1. Estimates of the molecular masses of these plasmids, calculated from their migratory distance in the gels are consistent with those calculated from contour length measurements.

The transformation of multiple resistance by the 90.3 Mdal (peak III) plasmid gave conclusive evidence that this was the plasmid carrying the aminoglycoside determinants and was the conjugable R plasmid in P. mirabilis 2138.

6. Nomenclature

To conform with other work being done in our laboratory and with uniform nomenclature as proposed by Novick et al. (83), the plasmids found in P. mirabilis 2138 were redesignated as follows: the 4.95 Mdal (peak I) plasmid has been renamed pFL10020; the 26.8 Mdal (peak II) plasmid has been renamed pFL10030; and the 90.3 Mdal (peak III) plasmid has been renamed pFL10010. The transconjugate E. coli J53-1(P1) has been redesignated as J53-1(pFL10010), the peak III transformants have been designated as C1200-1(pFL10010) and the peak I transformants as C1200-1(pFL10020). Transconjugates from other matings were designated by the recipient strain followed with the (pFL10010) designation.

In summary, then, the plasmid pFL10010 has been shown to confer

Figure 11. Agarose gel electrophoresis of plasmid DNA isolated from E. coli transformants. Peak III purified plasmid DNA, isolated from strain 2138, was used to transform E. coli C1200-1 to aminoglycoside resistance. Plasmid DNA isolated from carbenicillin and gentamicin resistant C1200-1 transformants was subjected to agarose gel electrophoresis as described in Figure 10. (A) Strain C1200-1 DNA. (B) Strain 2138 DNA. (C) Strain C1200-1 peak III transformant selected for gentamicin resistance. (D) Strain C1200-1 peak III transformant selected for carbenicillin resistance. (E) Strain C1200-1 peak I transformant selected for carbenicillin resistance. (F) Strain J53-1(pFL10010) transconjugate DNA.

A B C D E F

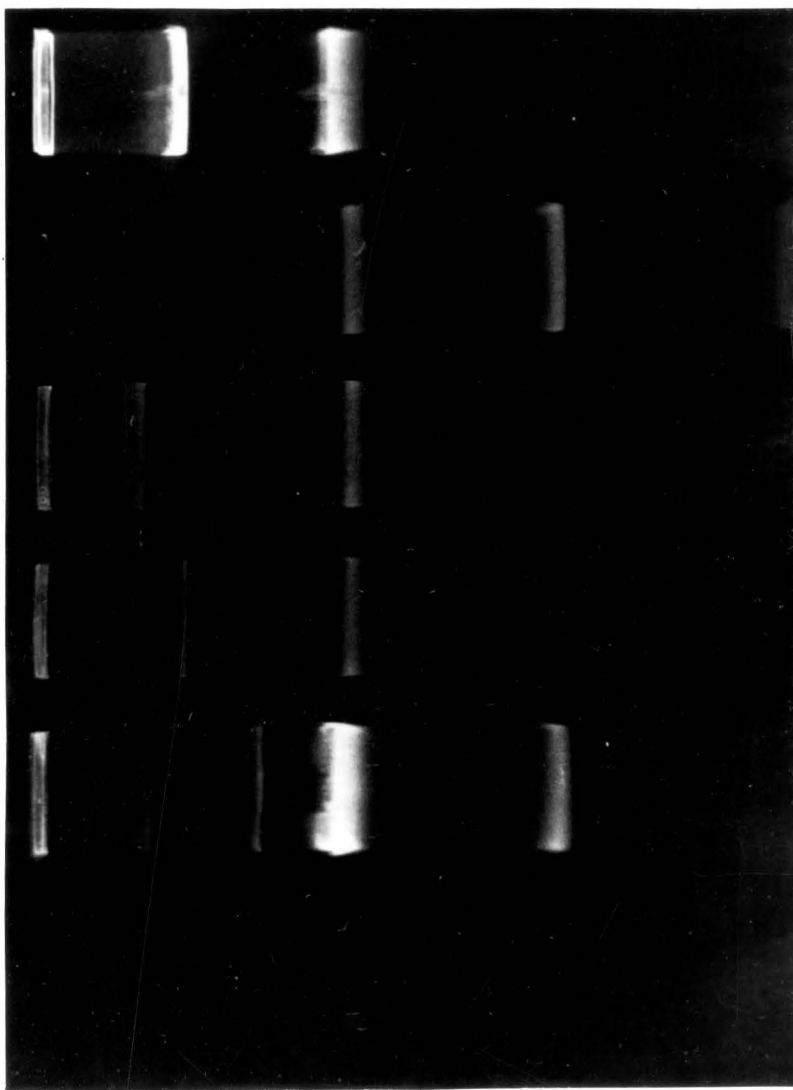
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on its host bacterium resistance to ampicillin, carbenicillin, gentamicin, tobramycin, kanamycin, streptomycin, and cephalothin. The small plasmid, pFL10020, confers resistance to ampicillin, carbenicillin and cephalothin only upon its host cell. Of the four tested no resistance traits have been associated with the plasmid pFL10030.

7. Host Range Studies

The prominence of multiple drug resistance among gram-negative bacterial isolates from patients at Hines V.A. Hospital has become a formidable problem (65). To determine the possible role of pFL10010 in this scheme, the host range of this plasmid was investigated. Standard conjugation experiments were performed using genetically characterized members of the Enterobacteriaceae and other gram-negative genera as recipient strains. If broth matings failed to demonstrate transfer of the R plasmid from either strain J53-1 (pFL10010) or strain 2138 to the recipient strain, the filter method was employed as described (see Materials and Methods). Transconjugates were selected on minimal medium containing either gentamicin (50 g/ml) or carbenicillin (100 µg/ml) and the necessary auxotrophic requirements.

The matings and frequencies of conjugation are shown in Table VI. With the exception of Proteus mirabilis Pml5, both Cb and Gm resistance traits were transferred to the recipients at approximately the same frequency. The R plasmid was readily transferred from strain J53-1(pFL10010) to E. coli C1200-1, Enterobacter cloacae 23355,

Table VI.

Host Range Studies

Donor strain ^a	Recipient strain	Transfer frequency ^b	
		Cb selection	Gm selection
2138	<u>E. coli</u> J53-1	1.3×10^{-2}	1.5×10^{-2}
	<u>E. coli</u> C1200-1	1.3×10^{-2}	1.1×10^{-1}
	<u>P. aeruginosa</u> PA038	ND ^c	4.5×10^{-7}
	<u>P. vulgaris</u>	$<10^{-8}$	$<10^{-8}$
J53-1(pFL10010)	<u>E. coli</u> C1200-1	3.0×10^{-7}	1.8×10^{-7}
	<u>E. colacae</u> 23355	4.4×10^{-5}	5.1×10^{-5}
	<u>S. marcescens</u> 8100	3.1×10^{-7}	3.1×10^{-7}
	<u>K. pneumonia</u> 27730	9.3×10^{-8}	9.3×10^{-8}
	<u>S. flexneri</u> Sfl	3.2×10^{-6}	3.2×10^{-6}
	<u>P. mirabilis</u> Pm15	2.2×10^{-5}	9.3×10^{-5}
	<u>S. typhimurium</u> TA1637	2.0×10^{-8}	1.0×10^{-8}
	<u>E. coli</u> CSH-K-12	$<10^{-8}$	$<10^{-8}$
	<u>P. vulgaris</u> 6380	$<10^{-8}$	$<10^{-8}$
	<u>P. aeruginosa</u> PA038	$<10^{-8}$	$<10^{-8}$
	<u>A. tumefaciens</u> B6-806	$<10^{-8}$	$<10^{-8}$
	<u>A. tumefaciens</u> 15955	$<10^{-8}$	$<10^{-8}$

^a All matings using strain 2138 were performed by the filter technique. Those matings using strain J53-1(pFL10010) as donor were done in broth cultures. (See Materials and Methods section).

^b Expressed as number of transconjugate colony forming units (CFU) per ml divided by number of input donor CFU per ml.

^c ND - not done.

Serratia marcescens 8100, Klebsiella pneumonia 27730, Shigella flexneri Sfl, Proteus mirabilis Pml5 and Salmonella typhimurium TA1637 with frequencies of conjugation ranging from 10^{-5} to 10^{-8} . Transfer of the R plasmid was not detected in matings between J53-1(pFL10010) and E. coli CSH K12, Proteus vulgaris 6380, Pseudomonas aeruginosa PA038, or Agrobacterium tumefaciens strains B6-806 or 15955.

Attempts to transfer the R plasmid from strain 2138 could not be achieved without the use of membrane filters to concentrate the donor and recipient cells. Using the filter method, the R plasmid was successfully transferred from strain 2138 to E. coli J53-1, E. coli C1200-1, and Pseudomonas aeruginosa PA038. Carbenicillin resistance only was transferred to Proteus mirabilis Pml5, and no transfer of antibiotic resistance could be demonstrated with the Proteus vulgaris 6380 recipient. Most significantly, the transfer of the R plasmid to P. aeruginosa PA038 expanded the host range of this plasmid beyond the Enterobacteriaceae. However, the plasmid was not transferred to the strains of Agrobacterium which have been shown to accept other broad host range R plasmids (96, 108).

Transconjugates from each mating were examined for their antibiotic resistance phenotype using the disk diffusion technique previously described. A minimum of 10 clones from each mating were tested and the results are given in Table VII. As indicated, some of the recipient strains are inherently resistant to certain antibiotics. For example, Serratia marcescens 8100 and Klebsiella pneumonia 27730 are resistant to ampicillin, P. mirabilis Pml5 is resistant to tetracycline,

Table VII.

Resistance patterns^a of donor, recipient and transconjugate strains.

Strain ^b	Antibiotic ^c	No. of isolates ^d
<i>P. mirabilis</i> 2138	Ap Cb Gm Tm Km Sm Cs Cl Nm Tc	1
<i>E. coli</i> J53-1(pFL10010) ^e *	Ap Cb Gm Tm Km Sm Cl Rm	1
<i>E. coli</i> C1200-1	Rm	1
C1200-1(pFL10010)	Ap Cb Gm Tm Km Sm Cl Rm	10/10
<i>P. mirabilis</i> Pml5	ND Tc Rm	10/10
Pml5(pFL10010)	Ap Cb Gm Tm Km Sm ND ^f Tc Rm	15/15
<i>P. aeruginosa</i> PA038*	Ap Km Sm Cs Cl Tc	10/10
PA038(pFL10010)	Ap Cb Gm Tm Km Sm Cs Cl Tc	12/12
<i>E. cloacae</i> 23355		10/10
23355(pFL10010)	Ap Cb Gm Tm Km Sm Cl	10/10
<i>S. marcescens</i> 8100	Ap Cl	10/10
8100(pFL10010)	Ap Cb Gm Tm Km Sm Cl	10/10
<i>S. typhimurium</i> TA1637		10/10
TA1637(pFL10010)	Ap Cb Gm Tm Km Sm Cl	10/10
<i>K. pneumonia</i> 27730	ND	5/5
27730(pFL10010)	ND Cb Gm Tm Km Sm Cl	10/10
<i>S. flexneri</i> Sf1		5/5
Sf1(pFL10010)	Ap Cb Gm Tm Km Sm Cl	10/10

a All strains were tested for resistance to Ap, Cb, Gm, Tm, Km, Sm, Cs, Cl, Nm, Tc and Rm by the disk diffusion method (69).

b Transconjugate strains resulting from matings using strain 2138 as donor are indicated by*. All other transconjugate strains resulted from matings with strain J53-1(pFL10010).

c Abbreviations as described in Table III.

d Number of clones having this phenotype per total number of clones tested.

e *E. coli* J53-1(pFL10010) was formerly designated as *E. coli* J53-1(P1).

f ND - not done.

and P. aeruginosa PA038 is resistant to ampicillin, kanamycin, streptomycin, colistin, cephalothin and tetracycline. As seen in the original mating between strains 2138 and J53-1, no transfer of resistance to either neomycin or tetracycline was detected. A resistance phenotype of Ap, Cb, Gm, Tm, Km, Sm, and Cl was transferred en bloc to the transconjugate strains. Although the recipient strain P. aeruginosa PA038 was resistant to five of the antibiotics, the transconjugate clones showed additional resistance to Cb, Gm, and Tm indicating that transfer of pFL10010 had occurred.

In the mating between strain 2138 and P. mirabilis Pml5 resistance to the beta-lactam antibiotics only was detectable. However, the transconjugates resulting from the mating of strains J53-1 (pFL10010) and Pml5 demonstrated resistance to the aminoglycoside antibiotics as well (Table VII). It is possible in matings with strain 2138 that the smaller plasmid pFL10020 could have been mobilized by the transfer of pFL10010, but this would not be detectable by phenotype.

8. Plasmid Stability

It was noted during routine passage of stock cultures that some clones not maintained on medium containing gentamicin lost their resistance to that antibiotic. Plasmid stability in several transconjugates was checked by passing clones daily onto fresh NA plates and testing at two week intervals for Gm and Cb resistance. One hundred clones of each strain were tested, and the results are shown in Table VIII. From this data it is evident that the stability of the plasmid

Table VIII

Stability of plasmids in transconjugate strains.^a

Strain ^b	Test medium: ^c No. passages:	Subclones showing growth (%)								
		14	28	Na 42	14	Cb 28	42	14	Gm 28	42
<i>P. mirabilis</i> 2138		100	100	100	100	100	100	100	100	100
<i>P. mirabilis</i> Pm15 (pFL10010)		100	100	100	100	100	100	52	25	0
<i>E. coli</i> J53-1 (pFL10010)*		100	100	100	100	100	100	57	6	0
<i>P. aeruginosa</i> PA038 (pFL10010)*		100	100	100	95	80	40	97	0	0
<i>E. coli</i> C1200-1 (pFL10010)		100	100	100	100	100	100	100	100	100
<i>S. flexneri</i> Sf1 (pFL10010)		100	100	100	100	100	100	100	100	100
<i>S. marcescens</i> 8100 (pFL10010)		100	100	100	26	20	0	0	0	0
<i>K. pneumonia</i> 27730 (pFL10010)		100	100	100	100	100	100	66	50	50
<i>E. cloacae</i> 23355 (pFL10010)		100	100	100	100	100	100	100	100	100
<i>S. typhimurium</i> TA1637 (pFL10010)		100	100	100	100	100	100	100	100	100

^a All strains passed on nutrient agar.^b Strains derived from matings with strain 2138 are indicated by *. All other strains derived from matings with J53-1 (pFL10010).^c NA - nutrient agar, Cb - nutrient agar containing carbenicillin (100 µg/ml), Gm - nutrient agar containing gentamicin (50 µg/ml).

is a function of the strain in which it is harbored as well as the nature of the plasmid itself. Gentamicin resistance, carried only on the large plasmid, pFL10010, was unstable in five transconjugate strains: P. mirabilis Pm15 (pFL10010), E. coli J53-1(pFL10010), P. aeruginosa PA038(pFL10010), S. marcescens 8100(pFL10010) and K. pneumonia 27730(pFL10010). Carbenicillin resistance which is carried on both plasmids, pFL10010 and pFL10020, was lost in only two strains: P. aeruginosa PA038(pFL10010) and S. marcescens 8100(pFL10010). In both cases loss of Cb resistance was concomitant with the loss of Gm resistance. Disk diffusion testing further showed that those clones which had lost Gm resistance also lost resistance to the other aminoglycoside antibiotics.

CHAPTER IV

DISCUSSION

Infections of the urinary tract with strains of Proteus mirabilis are common and second only to Escherichia coli infections in the frequency with which they occur (97). Due to their special predilection for the upper urinary tract, Proteus infections cause much greater kidney damage than E. coli infections which are usually limited to the bladder. Proteus mirabilis is now recognized as a frequent nosocomial pathogen and has been implicated in several nosocomial outbreaks of infection (9). The increasing number of Proteus clinical isolates resistant to aminoglycoside therapy has become a medical problem (87).

Yoshikawa et al. (116) have undertaken an extensive study of the antimicrobial susceptibility pattern of Proteus isolates, and have shown an increasing occurrence of resistant Proteus strains. Due to the cross reactivity of aminoglycoside inactivating enzymes (78, see Introduction), the efficacy of treatment with those antibiotics has been greatly reduced. Since there are only two known enzymes that inactivate Amikacin, it is now considered the drug of choice for Proteus infections. However, the effectiveness of Amikacin may be decreasing with the increased number of resistant isolates (116).

In this study a Proteus mirabilis clinical isolate (strain 2138) was shown to be multiply resistant to Ap, Cb, Gm, Km, Nm, Sm, Tm, Cs, Cl and Tc (Table III). Analysis of the MBC of these drugs reveals high levels of resistance, well in excess of obtainable patient serum levels for each antibiotic. Such high levels of antibiotic resistance, as

seen in strain 2138, are usually indicative of R plasmids which constitutively synthesize antibiotic inactivating enzymes (78).

The genetic evidence from matings with E. coli clearly show that the resistance phenotype is due, in part, to R plasmids (Table III). Resistance to tetracycline was not transferrable and probably reflects the inherent chromosomal resistance to this antibiotic which is characteristic of the species (30). It is unlikely that resistance to neomycin and tetracycline exist on the large plasmid but are not phenotypically expressed. Were this the case expression of antibiotic resistance to those drugs should have been seen in at least some of the transconjugate strains. Resistance to tetracycline and neomycin was not detected in any of the transconjugants using the J53-1(pFL10010) donor. Given the cross-reactivity of aminoglycoside inactivating enzymes, it is interesting to note that neomycin resistance was not transferred along with resistance to five other antibiotics.

There must be several antibiotic inactivation enzymes in strain 2138, including a beta-lactamase and at least two aminoglycoside inactivating enzymes. The most common beta-lactamases associated with plasmids and transposons of gram-negative origin are the TEM-like enzymes. Unlike the O type, the TEM beta-lactamases have a broader general activity on penicillins and cephalosporins (39, see Introduction), such as that observed in strain 2138. A single aminoglycoside inactivating enzyme could account for Gm-Tm-Km inactivation (see Introduction). Analysis of the substrates of the inactivation enzymes reveals that more than one enzyme is known to effect Gm-Tm-Km in-

activation without inactivating Nm. No enzyme is known to inactivate any of these three aminoglycosides exclusively, although strain 2138 may carry two or three enzymes which inactivate different combinations of enzymes. Since streptomycin inactivating enzymes are specific and do not cross react with other aminoglycosides, strain 2138 must also contain a streptomycin inactivating enzyme. The mechanism of resistance to colistin (Polymixin E) is unknown but it appears not to be plasmid coded. Several gram-negative species, including Proteus, are known to be insensitive to this drug, and the resistance is thought to be chromosomally mediated (30).

Physical-chemical evidence of the existence of extrachromosomal DNA in strain 2138 and its transconjugate strain J53-1(pFL10010), came from isolation of plasmid DNA in CsCl-EtBr density gradients (Figure 1). Initial isolations using the Helinski lysis procedure led to the detection of two plasmid species in strain 2138, but only one in strain J53-1(pFL10010). Contour length measurements of electronmicrographs showed the molecular mass of the Proteus plasmids to be approximately 5 and 27 megadaltons, whereas the plasmid isolated from the E. coli transconjugate was 90 megadaltons. Early speculations that the 90 Mdal plasmid might be dissociated in the Proteus species, or that the transconjugate plasmid was a trimer of the 27 Mdal plasmid were disproven. The two plasmids isolated from strain 2138 failed to transform E. coli C1200-1 to aminoglycoside resistance. However, resistance to the penicillins was transferred by the smaller plasmid.

Analysis of neutral and alkaline sucrose gradients of strain 2138

plasmid DNA isolated by the Helinski method led to the conclusion that a third, larger plasmid existed in that species. The Currier plasmid isolation procedure was employed for its ability to isolate large plasmids without breakage (15). Through use of this technique, a 90 megadalton plasmid was isolated from strain 2138. Comparison of contour length measurements and migrations in agarose gels revealed that the large plasmid found in strain 2138 was the same size as the plasmid isolated from the transconjugate strain J53-1(pFL10010).

The difficulty in isolating the 90 Mdal plasmid from strain 2138 using the Helinski partial lysate technique was most probably due to its size and to the susceptibility of Proteus to the detergents used to lyse the cells. Although the plasmid was recovered from strain J53-1 (pFL10010) using this method, the yield was poor. The Helinski method is based on a partial lysis of the bacterial cell wall which allows the leakage of small plasmid molecules while retaining the larger chromosome. The Proteus cells were not highly susceptible to the lysis which explains why only the small molecules were recovered. The E. coli cells were extremely sensitive to the lysis which often went to completion allowing the large plasmid, along with the chromosome, to leak out. The alkaline sucrose gradients showed that some 90 Mdal plasmid DNA was present in the Proteus lysates but its detection was initially masked by the high yields of the smaller plasmids. The Currier method of plasmid isolation involves a total lysis of the cells with subsequent separation of the chromosome from plasmid DNA. This method was designed for isolation of large plasmids (in excess of 60 Mdal) and

proved to be effective in this study. Yields of pFL10010 plasmid DNA from both strains 2138 and J53-1(pFL10010) were significantly increased using this method.

Conclusive evidence that the 90 Mdal pFL10010 plasmid was the R plasmid responsible for aminoglycoside resistance comes from the transformation of E. coli C1200-1 using highly purified and size-fractionated plasmid DNA isolated from strain 2138. Although the 5 Mdal plasmid pFL10020 was shown to transform strain C1200-1 to penicillin resistance, only the pFL10010 appeared capable of transforming strain C1200-1 to aminoglycoside resistance (Table V). Cells transformed with pFL10010 DNA were found to contain only this plasmid (Figure 1).

The host range studies (Table VI) further revealed that the pFL10010 plasmid could be transferred in vitro to several strains of the Enterobacteriaceae and also to the non-Enterobacteriaceae species Pseudomonas aeruginosa PA038. However, the frequency of transfer of the plasmid was low, and could only be achieved through use of the filter mating technique. Concentration of donor and recipient cells by vacuum aspiration onto millipore filters increases cell to cell contact thus enhancing the probability of plasmid transfer. Employment of this technique did not prove successful in all cases. No detectable plasmid transfer was seen between strains 2138 and Proteus vulgaris 6380, and only carbenicillin resistance was transferred to Proteus mirabilis Pml5. In general, Proteus species are considered poor donors and recipients of R plasmids (24) and several R plasmids are known to undergo dissociation in this species (see Introduction).

However, one would expect an intragenic transfer of R plasmids to succeed. The fact that transfer to P. vulgaris 6380 using strain J53-1(pFL10010) as donor also failed is probably a reflection of the inherent properties of that strain and is consistent with other experiments involving this strain (24). It is interesting that P. mirabilis Pml5 received only the carbenicillin resistance trait when mated with strain 2138, yet received both Cb and aminoglycoside resistance traits when mated with J53-1(pFL10010). In plasmid stability experiments gentamicin-resistant clones of strain Pml5(pFL10010) not maintained on Cm-containing medium lost their resistance to Gm while retaining Cb resistance (Table VIII). It appears that the R plasmid is not stably maintained by this strain, and that the Cb-resistance trait can maintain independent existence in this strain. Since the pFL10020 plasmid could not have been received in matings with J53-1(pFL10010), it seems likely that the Cb-resistance determinant on the pFL10010 plasmid has transpositional activity, and has integrated into the chromosome or some indigenous plasmid of strain Pml5(pFL10010).

In addition to P. vulgaris 7380, other strains failed to acquire the R plasmid in broth matings with J53-1(pFL10010). These include matings with the non-related species of Agrobacterium tumefaciens and P. aeruginosa PA038. It is interesting that the intragenic mating between J53-1(pFL10010), a K12 derivative, and E. coli CSH-K12 also failed. Successful transfer of the R plasmid was achieved from strain J53-1(pFL10010) to E. coli C1200-1, but only at low frequency of transfer (10^{-7}). It is possible that transfer to strain CSH-K-12 may have

occurred, but was below the detectable limits of the experiment (10^{-8}), and would have been enhanced through use of the filter mating technique. It is possible that phage λ could have been liberated from J53-1 (pFL10010) donor and killed the CSH-K12 recipient which does not contain the phage. However, were this the case, one would have expected strain C1200-1, which also lacks the phage, to be killed. It was later found that the stock cultures of strain CSH-K-12 may have been contaminated.

The promiscuity of the R plasmid pFL10010 is shown by its ability to transfer to the non-Enterobacteriaceae species Pseudomonas aeruginosa. Since strain PA038 is intrinsically ampicillin resistant, gentamicin selection alone was used for the mating. The disk diffusion tests did show that the transconjugate strain PA038(pFL10010) did acquire other resistance traits encoded by pFL10010 (Table VII). Broth matings between strain J53-1(pFL10010) and PA038 did not yield transconjugates. However, successful mating was achieved between strains 2138 and PA038 when the filter disk technique was employed. This indicates that Pseudomonas is a poor recipient and transferability is only detected when enhanced by the filter technique. Of clinical significance beyond the broad host range of the R plasmid, is its ability to transfer at high frequencies to the pathogenic species of Shigella, Salmonella and Klebsiella. A multiply resistant plasmid with these capabilities could present a formidable medical challenge.

As previously mentioned, the R plasmid was not maintained in all transconjugate clones. As in the case of P. mirabilis Pml5(pFL10010),

the strains E. coli J53-1(pFL10010) and K. pneumonia 27730(pFL10010) lost Gm resistance while retaining Cb resistance. Resistance to both Gm and Cb was lost in clones of P. aeruginosa PA038(pFL10010) and S. marcescens 8100(pFL10010). It is possible that in matings with strain 2138 the smaller plasmid pFL10020 could be mobilized during transfer of pFL10010. The transconjugate strain having pFL10020 would continue to be Cb resistant even if it lost the larger plasmid. Since strain 27730(pFL10010) is the result of a mating using J53-1(pFL10010) as donor, retention of the Cb resistance trait cannot be explained by the presence of the plasmid pFL10020 in the transconjugate strain.

It appears that the R plasmid pFL10010 contains a Tn~~1~~-like transposon capable of either independent existence or chromosomal integration. The size of the Proteus plasmid pFL10020 is consistent with known Tn1-like transposons (25) which raises the possibility that this small plasmid may be a dissociated r-determinant derived from the pFL10010 plasmid.

In a hospital environment where the increased use of prophylactic antibiotics provides a selective pressure, the evolution of multiply-resistant R plasmids is encouraged. Given the promiscuity of the plasmid pFL10010, one might envision its evolution to multiple resistance by acquiring resistance traits singly as it was transferred among various species of bacteria. The possibility of transpositional activity of pFL10020 makes this suggestion credible. The fact that the R plasmid did not transfer to P. vulgaris 6380 and only to P. mirabilis Pm15 at low frequency suggests that Proteus may not be the original

host of the R plasmid, and was just the most recent of many hosts in its evolution.

CHAPTER V.

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

20 November, 1980

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