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INFECTIOUS MULTIPLE DRUG RESISTANCE IN THE ENTEROBACTERIACEAE

ANNUAL PROGRESS REPORT

by

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Preface

Over the past years our work under this contract has taken on a definite cyclic pattern. We begin with a clinical or epidemiological observation concerning microbial antibiotic resistance or virulence. We next attempt to define the basis for the observation in precise genetic and molecular terms and, finally, we attempt to exploit these 'basic findings' in an applied way to ask further epidemiological questions on drug resistance or pathogenesis. Last year we reported the application of molecular genetics to the question of epidemiological typing of R-factors from epidemic strains and uncovered a novel mechanism by which antibiotic resistance genes become disseminated from plasmid to plasmid. This year we report the genetic and molecular basis for this dissemination process and give preliminary results on how it can be directly applied to the study of pathogenesis and its potentiality for the preparation of vaccine strains.

As in previous years, the students in my laboratory have made the most significant contributions to this research program. I wish to particularly single out a graduate student, Frederick Heffron and an undergraduate student, Craig Rubens for their tireless, innovative labors.

INTRODUCTION

Plasmids are extrachromosomal DNA elements of bacteria which may be transmitted directly or indirectly among different species of bacteria. Plasmids are known to carry a variety of genes that may be of selective advantage to the host bacterial cell under certain selective environmental conditions. The best known example of bacterial plasmids are the R factors which carry genes determining resistance to antimicrobial agents. Indeed, resistance to all of the antibiotics used therapuetically have been identified on R-plasmids and individual R-plasmids may carry resistance for as many as 10 distinct antibiotics. Although R plasmids have been known since 1958, until recently little has been known about the mechanisms involved in their formation or the origin of their carried drug resistance genes. It is known that the mechanism of antibiotic resistance mediated by R-plasmids involves enzymes known to either hydrolyze or modify the antibiotic to a relatively innocuous form. Moreover, it has been shown that similar enzymes are found only in species of Streptomyces which produce antibiotics, but it has not been possible to mutate any drug sensitive enteric species to drug resistance and find analogous drug inactivating enzymes. The speculation has been, therefore, that the drug inactivating enzymes are encoded by genes which were initially found in Streptomyces sp, but have somehow found their way onto the plasmids of bacteria. Despite this speculation there has been no experimental data to definitively show the origin of the drug resistance genes found on R-plasmids. Nevertheless, there has been a good deal of recent concentration on the molecular nature of the antibiotic resistance genes to find a clue to their origin(s).

One of the most striking features of our previous work under this contract has been the observation that quite different R-plasmids present in widely diverse groups of microorganisms often carry resistance genes which confer the same

mechanism of resistance. For example, R-mediated resistance to penicillins and cephalosporins is universally associated with the elaboration of a penicillinase (β-lactamase). Two general classes of R-mediated β-lactamase are now recognized. The TEM β-lactamase which has a high relative activity on benzyl penicillin and cephaloridine substrates but low activity against isoazolyl penicillins such as oxacillin and methicillin and O β-lactamase which is of lower absolute activity but can appreciably hydrolyze all penicillin and cephalothin derivatives. TEM is the most common R-mediated β -lactamase and is found on a wide variety of naturally occurring plasmids. Moreover, regardless of the species of origin or the geographical source of the R-plasmid, the TEM β-lactamase proteins have been found to be virtually identical- the only differences being in one or two amino acid substitutions in a 22,500 dalton protein. In our last annual report we presented unequivocal evidence to show that the TEM β-lactamase gene of R plasmids is situated on a 3.2×10^6 dalton segment of DNA which is capable of migration (gene translocation) from one plasmid to another. Not only could we show that the TEM gene could be translocated but also that the identical sequence of TEM DNA was present on naturally occurring R plasmids of the FI, FII, N, I, O, C, P, W and X incompatibility groups. This DNA sequence was not found on naturally occurring R-plasmids of the same incompatibility groups which did not specify ampicillin resistance or specified the O β-lactamase. While the evidence for the translocation of the TEM β-lactamase on a sequence of DNA seemed clear enough, the precise genetic mechanism(s) mediating translocation remained unclear. In the following sections we report our progress over the past year in elucidating the mechanism of the translocation of ampicillin resistance and its implications to the study of the epidemiology of R plasmids in enteric and other gram negative bacteria.

RESULTS

A. A Laboratory Model to Study the Translocation of Ampicillin Resistance

Our initial discovery of the translocation of ampicillin resistance from plasmid to plasmid was based on a) studies of DNA-DNA homology between plasmids as well as b) the demonstration that a plasmid receiving ampicillin resistance by "recombination" showed an increase in molecular weight of about 3×10^6 daltons. If one wished to more precisely define the translocation of ampicillin resistance genes, therefore, it was necessary to devise a laboratory model in which this process could be directly monitored and its molecular nature and specificity discerned. In essence, what we required was a well characterized plasmid which could serve as an acceptor of the TEM DNA sequence, hereafter called TnA, as well as a means to directly examine the molecular and genetic consequences of the acquisition of TnA. Fortunately, in past years we had studied in some detail the genetic and molecular properties of naturally occurring non-conjugative plasmids, 5.5×10^6 daltons in size, which encoded for sulfonamide (Su) and streptomycin (Sm) resistance. One of these plasmids, RSF1010, was chosen by us as an acceptor of TnA because of technical reasons which will become apparent shortly.

The isolation of RSF1010 carrying TnA was not difficult. An *E. coli* K-12 strain carrying RSF1010 was infected with a larger conjugative R plasmid carrying ampicillin resistance (that is the TnA DNA sequence). We have employed the 65×10^6 dalton R-plasmid, R1*drd*19 (Su Sm Cm Km Ap) in most experiments (Other conjugative plasmids, RP4 (Km Ap) and R6K (Sm Ap) have also been used successfully). The plasmid DNA from this strain was isolated as covalently closed molecules by dye buoyant density equilibrium centrifugation and then sedimented through a 5 to 20% neutral sucrose gradient. R1*drd*19 molecules sediment at 75S, while the RSF1010 molecules sediment at 26S. The two molecular species are quite easily separated, therefore, by collecting 5-drop fractions from the sucrose gradient through a hole punched in the bottom of the tube. The individual

fractions across the gradient were then employed as a source of DNA to transform $CaCl_2$-treated E. coli. This is the crux of the method. $CaCl_2$-treated E. coli can take up circular plasmid DNA which can thereafter replicate and be stably maintained. If one selects for Ap^r transformants across a gradient prepared with an artifical mixture of R1drd19 and RSF1010 DNA, Ap^r transformant cells are only isolated from the region of the gradient known to contain R1drd19 molecules. However, when we transformed cells across a gradient with plasmid DNA isolated from a strain that has harbored both R1drd19 and RSF1010, two distinct regions of the gradient give rise to Ap^r transformants. One of these corresponds to the region of the gradient known to contain R1drd19 molecules; the other region from which Ap^r cells can be isolated was found sedimenting just ahead of normal RSF1010 molecules (about 32S). The number of Ap^r transformants isolated from the 35S region was small and equivalent to only about 0.01% of the total RSF1010 population. Sixteen of the Ap^r transformants isolated from the 32S region were found to be $8.7 \pm 0.2 \times 10^6$ daltons.

RSF1010 can be quantitatively converted to linear molecules of unit length by treatment with the restriction endonuclease, EcoRI. This enzyme places a staggered cut in both strands of a DNA molecule bearing a specific hexanucleotide sequence. The distribution of this specific sequence varies from plasmid to plasmid ranging from 1 for RSF1010 to 17 for R1drd19. The 8.7×10^6 dalton plasmids isolated from the 32S region all contained only a single EcoRI site so, like RSF1010, they would be quantitatively converted to linear molecules. Mixtures of normal linear RSF1010 DNA and the DNA from the 8.7×10^6 dalton DNA were heteroduplexed and examined in the electron microscope. Figure 1 (top panel) shows that a heteroduplex formed between 1 strand of RSF1010 and 1 strand of the 8.7×10^6 transformant DNA form a perfectly matched double-stranded DNA molecule except for a single well-defined loop of single stranded DNA. Thus, the

Ap^r transformants which are 8.7×10^6 daltons in size contain all of the 5.5×10^6 RSF1010 genome plus a 3.2×10^6 dalton insertion of DNA. This insertion (extra) DNA could be shown by DNA-DNA homology experiments to correspond to the TEM DNA (TnA) sequences as described in last years report. Thus, TnA emerges as a 3.2×10^6 dalton segment of DNA which includes the genes specifying the TEM β -lactamase which can migrate from plasmid to plasmid. It is also noteworthy to re-emphasize the fact that this process can be observed if two plasmids merely coexist within the same cell- no 'heroic' manipulations are required to force the migration of TnA. Another important feature of TnA translocation can be seen in heteroduplexes similar to that shown in Figure 1. The single-stranded TnA loop is seen to emanate from a short (about 150 base pair, 100,000 dalton stalk). This stalk is part of TnA and corresponds to an inverse repeated sequence of DNA which flanks the TnA sequence. As noted later we suppose it is these sequences (or a portion thereof) that are critical to the excision of TnA and its subsequent integration into another plasmid. Of course, since all of the Ap^r transformants possessed a molecular weight of 8.7×10^6 dalton, it seems clear that TnA is a very precise sequence of TNA (see also below).

The use of EcoRI cleaved linear molecules for examination in electron microscope heteroduplex experiments permitted us to visualize the TnA sequence and to determine that more than one site was involved. However, to precisely map the specificity of insertion it was necessary to devise a method to distinguish the 'right-hand' end of RSF1010 from the 'left-hand' end of the molecule. Fortunately, this was not a formidible technical problem. As noted in earlier reports, many non-conjugative Su Sm plasmids related to RSF1010 can be isolated from clinical isolates. One of these, R684, was found to be only slightly larger (about $0{\cdot}7 \times 10^6$ daltons). As shown in Figure 1 (middle panel) a heteroduplex molecule between RSF1010 and R684 shows identity except for a single insertion loop about 35% from one end. The EcoRI end closest to this loop is

designated the right-hand end of the molecule. Consequently, in order to precisely map the site of insertion of TnA it was simply necessary to heteroduplex RSF1010 Ap^r derivative with R684 (Fig 1 bottom).

B. The Specificity of TnA Insertion

The translocation of TnA from R1drd19 to RSF1010 has been performed in two homogenic E. coli K-12 strains which differed only in bacterial rec functions. As shown in Table 1, a comparison of 200 recombinant plasmids from a recombination proficient host and 200 recombinant colonies from a recombination deficient host are not significantly different ($p > .05$ by unpaired T test). Thus, the translocation of TnA is independent of normal bacterial recombination functions and is analogous to that observed for the insertion of bacteriophage into the bacterial chromosome. Four distinct phenotypic classes of RSF1010 carrying TnA have been observed. The most common group was Ap^r Su^r Sm^r but all combinations of Su^r/Su^s and Sm^r/Sm^s were observed. An examination of each of the phenotypic classes revealed that all plasmids with an Su^s phenotype contained a TnA insertion within the right hand 10% of the molecule. In some cases insertion within this region also gave rise to Sm^s. The basis for the observation will be considered later. All plasmids of phenotype Ap^r Su^r Sm^s (the second most common phenotype) all showed insertion of TnA within the left-hand 10% of the molecule. Plasmids inhibiting the phenotype Su^r Sm^r Ap^r showed TnA insertions scattered over the molecule, although most were found in the interval from 10% to 30% from the left-hand end of RSF1010. In all, an examination of heteroduplex molecules from 30 independent isolates have shown 19 distinct sites of TnA insertion within RSF1010. Statistical analysis of insertion reveals two important points: 1) there is no significant difference between the specificity of insertion of TnA in rec^- and rec^+ hosts and 2) insertion of TnA is non-random. This later point indicates, therefore, that the insertion of TnA is dependent upon a specific short (probably a 4 or 5 nucleotide pair) sequence of DNA.

Insertion of TnA within the right hand 10% of the molecule gave rise to either Ap^r Su^s Sm^s or Ap^r Su^s Sm^r plasmids. One can explain the Ap Su^s Sm^s class as a case in which insertion of TnA has had a strongly polar effect so that not only is the structural gene into which it has been inserted inactivated but other genes within the same operon have been inactivated. This result would suggest that Su Sm are transcribed as a single polycistronic message but that insertion of TnA interrupts this operon with attendant polar effects. Of course the finding of Ap^r Su^s Sm^r plasmids argues against such an interpretation. Consequently, we further examined plasmids with insertions in the right hand 10% of RSF1010; all were Su^s but they varied in Sm^r. When an Ap^r Su^s Sm^r plasmid, B1M4, was heteroduplexed with a Ap^r Su^s Sm^s plasmid, A1M5, a surprising result was found which is illustrated in Figure 2(a). The heteroduplex shows two non-interacting TnA loops at precisely the same site on RSF1010. This finding is consistent with the conclusion that TnA can insert within RSF1010 with two orientations. In Figure 2(b) one can see a heteroduplex formed between B1M4 and another Ap^r Su^s Sm^s plasmid A_2M6. Again, one sees two non-interacting loops, although clearly the TnA insertion on each strand has occurred at two distinct sites. Further studies of this type have made it clear that insertion of TnA within RSF1010 with the orientation of A1M5 (orientation A) gives rise to plasmids with the phenotype Ap^r Su^s Sm^s (i.e. to a strongly polar effect), while insertion of TnA within RSF1010 with an orientation identical to that observed in B1M4 (orientation B) give rise to the Ap^r Su^s Sm^r phenotype (i.e. there is a promotor effect). In fact, in orientation B, the closer the insertion to the right-hand end of the molecule the higher the MIC for streptomycin (see Table 2). In all, 11 insertions into the right-hand end of RSF1010 have been examined, 6 from a rec^+ host, 5 from a rec^- host. All plasmids are Su^s indicating that the structural gene for resistance lies in this region. The 11 insertions fell into only 4 distinct sites. This finding again re-enforces the fact that insertion depends upon some non-random nucleotide sequence.

Moreover, the 11 insertions show that the differences in the orientation of insertion occur with about equal frequency and that the correlation between orientation and the Sm^r phenotype are as predicted from the heteroduplexes shown in Figure 2(a) and 2(b).

The differences in the orientation of TnA have also been independently confirmed by heteroduplex analysis between RSF1010 Ap^r derivatives and a ColE1 Ap^r derivative. RSF1010 and ColE1 show no measurable degree of homology so that any interaction between ColE1 Ap and RSF1010 Ap should be restricted solely to the TnA sequence. Examination of Figure 2(c), (d), (e) shows that, in fact, orientation of TnA be ascertained by orientation of the linear 'tails' with each other. In Figure 2(c), for example, ColE1 Ap vs B1M4 the short RSF1010 tail is seen aligned with a long ColE1 tail. In contrast, Figure 2(d) which is ColE1 Ap vs A_1M5 the long and short tails are aligned indicating a difference in the orientation of TnA. Figure 2(e) shows ColE1 Ap^r vs A_2M_6; this heteroduplex reveals that TnA is in the same orientation in A_1M5 and A_2M6 but different from that of B1M4 - the expected results. These heteroduplexes also reveal another facet. The TnA duplex region is linear. This suggests that integration occurs at a unique site on TnA delineated by the inverted repeat sequence. It should also be pointed out that the TnA insertions of ColE1 Ap and the RSF1010 Ap plasmids were from difference sources. One was derived from RP4 (a 34×10^6, 62% G + C plasmid from Pseudomonas) and the other from R1drd19. The finding of what appears to be a perfect duplex region indicates that either the TnA sequence is highly conserved or, alternatively, that it has been derived relatively recently (in the evolutionary sense). In point of fact, fine structure differences between the two TnA sequences have occurred since Hind III restriction digests show two cleavage sites for R1-derived TnA and three for RP4. However, the degree of mismatch is still quite low.

C. The Mode of Replication of RSF1010

If one grows RSF1010 to the mid-logarithmic phase of growth, deprives the host cell for thymine for 20 min., and adds a pulse of (^{3}H)-thymidine for 35 sec., the radioactivity is found banding between covalently closed and open circular molecules. This kinetic behavior is typical of that expected for replicative intermediates in which the molecules are partially covalently closed and partially open. Cleavage of such molecules with EcoRI yields linear molecules which contain a replication 'eye' and two unreplicated limbs. Since we had isolated derivatives of RSF1010 containing insertions of 3.2×10^6 dalton TnA in both the right hand end or left hand end of the molecule, it was possible to unequivocally map the origin and mode of replication for RSF1010, relative to one end of EcoRI cleaved molecules. These studies revealed that the origin of replication is located some 30% from the left-hand end of the molecule. Replication proceeds from this origin either unidirectionally or bidirectionally with about equal probability. It is not surprising that we have not isolated any RSF1010 derivatives of RSF1010 which show TnA inserted at 26-41%. Presumably, such an insertion, were it to occur would be a lethal event.

During the further examination of TnA insertion into RSF1010 several recombinant plamsids were isolated which had TnA inserted at 42% and 45% from the left-hand end of the molecule. Because of the proximity of these insertions to the origin of replication these plasmid classes were examined in some detail. One immediate effect noted was that plasmids inserted at 42% gave significantly higher yields of plasmid DNA. The explanation of this finding was immediately obvious when the MIC for antibiotics and the number of copies per cell of these plasmids were studied. As shown in Table 2, plasmid B2M4 with insertion at 42.1% from the left hand end of the molecule exhibited a 1.6 fold increase in Ap^r and a 4 fold increase in Sm^r. Moreover, as shown in Table 3, the number of copies of B2M3 and R-M1 with TnA inserted at 42.1 and 42%, respectively, was increased about 2

fold (as a minimal estimate). Surprisingly, plasmids with TnA insertions at 45.0 and 45.2% did not show either increased copy number nor increased MIC though they were only about 250 base pairs away from B2M3 and RM1 and only about 900 base pairs from the origin of replication. At any rate, insertions of TnA close to the origin of replication result in a modification of copy number with an attendant variation in phenotype. While this phenomenon is still under study, the data do shed some light upon the control of replication by plasmids and clearly indicate that copy number (the frequency of initiations) of plasmids is determined by a plasmid gene.

D. A Practical Exploitation of Ampicillin Translocation

Our concentration upon the molecular nature of TnA provide, we think, an important clue to the dissemination of drug resistance genes among R plasmids and offer a ready explanation for a number of epidemiological observations not the least of which is the recent appearance of Ap^r Haemophilus influenzae from cases of meningitis and epiglottistis. This latter finding will be pursued in considerable detail in our next annual report. It is also clear that the translocation of TnA can be exploited to map plasmid chromosomes and to inactivate specific gene sequences. We have, however, chosen to exploit TnA translocation to mark Ent and K plasmids. We have pointed out in earlier reports that these plasmids, which directly contribute to bacterial virulence, are difficult to study in the laboratory since they provide no selective advantage and their detection is dependent upon cumbersome tissue culture and animal models. It would, of course, be an advantage to be able to 'mark' such plamsids with a drug resistance marker in order to follow their 'travels' more easily. In the past contract period, we have accomplished this goal and developed a simple procedure which can be applied to clinical isolates with some success. In practice, one could transfer a plasmid carrying TnA to a toxigenic strain, isolate the plasmid DNA, sediment in sucrose and transform across the gradient as we did for RSF1010. This method

works in cases in which Ent and K plasmids have been transferred to E. coli K-12 but has several limitations. The major limitation is that Ent and K are large (usually 50 x 10^6 daltons or larger) and do not transform well. However, so long as Ent and K are transmissible, one can use an alternative method which requires nothing more difficult than plating on a selective medium with a special receptor strain. The principle is quite simple. The 4.2 x 10^6 dalton plasmid ColE1 cannot replicate in E. coli derivatives which are polI$^-$ nor can ColE1 derivatives which contain TnA. One can exploit this to modify a triparental mating to achieve the 'marking' of transmissible plasmids with Ap^r. The method is as follows. An Ent$^+$ (K$^+$) nalidixic sensitive strain is mixed with E. coli K-12 strain containing ColE1 Ap^r. Transmissible plasmids from the Ent (K) strain enter the ColE1 Ap^r strain after an overnight mating. This mixture is in turn mixed with an E. coli K-12 polI$^-$ nalidixic resistant strain and after an additional overnight incubation the entire mixture is plated on nutrient agar containing 25 ug/ml of nalidixic acid; and 50 ug/ml of carbenicillin. Colonies appearing on this medium contain transmissible plasmids carrying the TnA sequence. Such colonies can then be assayed for Ent, K, Hly or any other marker of interest and, subsequently, transferred to a host strain of choice. This method has now worked successfully with a number of isolates and we have been able to employ Ent Ap^r and K Ap^r derivatives in our work. Of course, this has greatly simplified their genetic analysis since one may now simply follow Ap^r rather than the tox, hemolytic or antigen marker.

This is but one example of the practical exploitation of translocation. Others including inactivation of viruelnce genes are currently being pursued and will be reported in due course.

Discussion

The significance of the translocation of ampicillin resistance is that it allows rearrangement and recombination of certain genes from heterologous backgrounds. Translocation is not limited to ampicillin but has now also been extended to encompass, tetracycline resistance, kanamycin resistance, chloramphenicol resistance, gentamicin resistance and trimethoprim resistance of R-plasmids. Thus, translocation provides a reasonable explanation for the accumulation of resistance genes by sex factors to form R-plasmids. The occurrence of transmissible plasmids which do not encode for any known function is common. We reported several years ago, for example, that some 42% of all E. coli strains carry some form of sex factor. Given the fact that antibiotic resistance genes migrate from plasmid to plasmid it is easy to see the evolutionary advantage that they would enjoy under conditions of antibiotic selection; there is no need for gross genetic homology normally required for recombination rather gene reassortment can occur if two plasmids simply occupy the same cell for a period of time. Given enough time and selection it is not hard to see how tandem or multiple insertions of translocation sequences would have occurred to form multiple drug resistance factors.

Translocation sequences such as TnA insert into and excise from DNA molecules in the absence of a functional bacterial rec system. The alternative recombinational mechanism employed by elements such as TnA appears to depend upon the recognition of a short common DNA sequence. In this sense insertion seems to mimic the specificity shown by restriction endonucleases which recognizes symmetrical nucleotide sequences composed of from 4-6 base pairs. Possibly the translocation sequence itself codes for such an enzyme. The critical sequence for both excision and insertion of TnA as well as analogous elements seems to be the presence of an inverted repeated DNA sequence flanking the major segment of DNA making up the translocation sequence. The presence of these

inverted repeated sequences are quite analogous of elements called IS sequences found in many microroganisms. E. coli K-12, for example, possesses several sequences called IS1, IS2, IS3 etc which range in size from 800 to 1800 nucleotide pairs. The IS sequences betray their presence by causing polar mutations. It is of considerable interest, therefore, to learn that the inverted repetitions flanking the tetracycline translocation sequence is identical to IS3. TnA like IS1, 2 and 3 are mutagenic when occurring in structural genes and polar when occurring within operons depending upon the orientation of insertion. This mutagenic property can be exploited to inactivate genes and prepare a functional map of plasmid genomes. One may also exploit the mutagenic effects of insertion to study virulence as, for example, the inactivation of plasmid-mediated enterotoxin genes.

As we have shown insertion of TnA is a relatively precise process since all plasmids receiving this sequence increase in mass by a precise amount. Our preliminary data suggest, however, that excision of TnA (and analogous elements) is imprecise and often leads in the permanent loss of host genetic information. So long as the two flanking inverted repetitions are conserved, however, the ability to insert is not lost, rather the excised fragment can circularize and return to its original form by intramolecular recombination between the two repeated ends. It is emphasized that circularized TnA sequences have not been detected and certainly if they exist their life-span would be short. Nor, is it yet established (or even likely) that TnA and other translocatable sequences can exist autonomously for any period of time. At any rate, the imprecise excision of TnA hold promise as a means to induce deletions in specific genes that can be exploited usefully. For example, if we once more use the insertion of TnA into an Ent gene for illustration it may be seen that selection for loss of TnA will result in the deletion of part of the Ent gene. By judicious selection, therefore, it would be perfectly feasible to be able to select a variant Ent

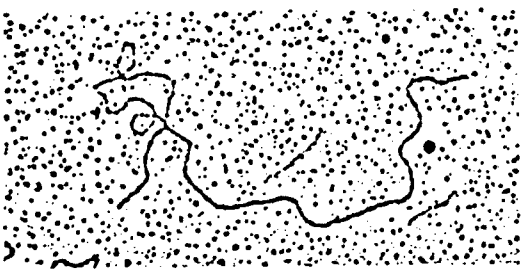
plasmid which produced an immunologically active protein devoid of toxic activity on animal cells. Likewise, one can force TnA into the chromosome of bacteria. Here again judicious selection could result in non-reverting avirulent mutants for use as vaccine strains. The applied applications of TnA are numerous if one takes advantage of its unique properties.

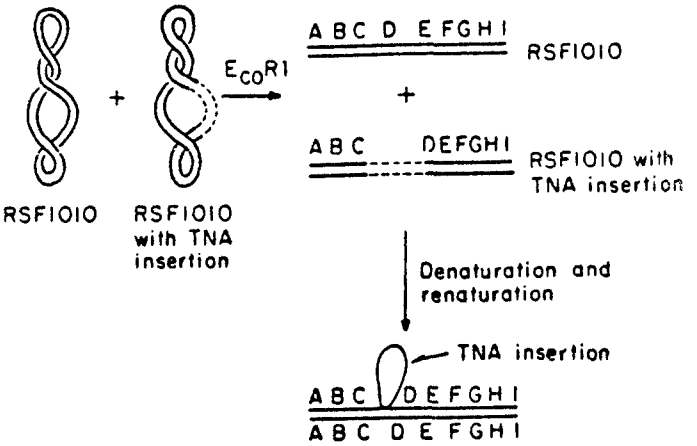
Translocation sequences such as TnA are powerful tools for genetic research since attachment of a resistance gene to a DNA molecule provides a readily identifiable genetic and physical marker. We have already exploited this feature for marking Ent and K plasmids. In the strict biological sense translocation is interesting in its own right as a biochemical mechanism affecting DNA-DNA interactions outside the usual forms of recombination. Nor should one overlook the fact that inverted repetitious DNA in eukaryotes are common and the study of similar elements in prokaryotic species can add to our fund of knowledge about possible mechanisms operating in many groups of living things.

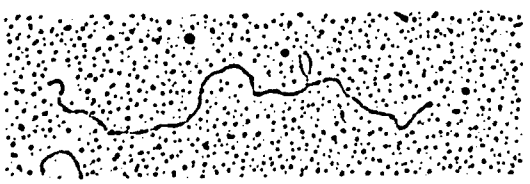
Finally, it is important to recognize the epidemiological implications of the discovery that antibiotic resistance genes of R-plasmids can migrate from molecule to molecule. This evolutionary flexibility is an enormous advantage. We can no longer think simply of the entension of whole plasmids frcm cell to cell. Rather, we must now think in terms of an enormous reservoir of genes available to insert into any DNA species. It has been clear that the stable direct extension of plasmid species has its boundaries. Thus, if one attempts for example, to transfer most R-plasmids of E. coli to Vibrio cholerae one finds that the plasmid per se is unstable. Yet, we now know that stable inheritance is not so much the important criterion as is the presence of a translocatable antibiotic sequence on the E. coli plasmid and a suitable recipient plasmid indigenous in Vibrio. It seems that this phenomenon was at work in the development of the R plasmids recently found in Haemophilus influenzae. Given the large reservoir of plasmids within bacterial species that could serve as acceptors of

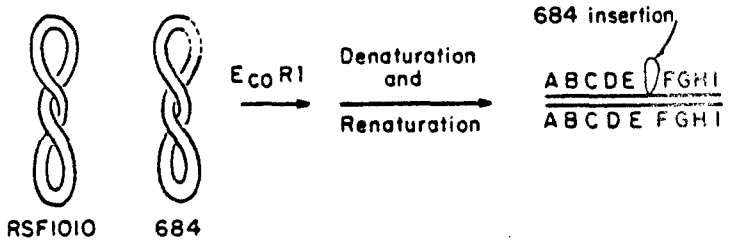
translocatable sequences as well as the reservoir of resistance genes (as, for example, in domestic animals fed medicated feeds for growth promotion) it probably is fair to conclude that the extension of drug resistance to bacterial species has few bounds given the time and appropriate selective conditions. Certainly, translocation of antibiotic resistance permits us to better understand the evolution of existing R-plasmids.

Figure 1. Method of Determing the Site-Specificity of TnA into a Recipient Plasmid Species.




RSF1010
RSF1010 with TNA insertion
EcoR1
A B C D E F G H I
RSF1010
A B C D E F G H I
RSF1010 with TNA insertion
Denaturation and renaturation
TNA insertion
A B C D E F G H I
A B C D E F G H I




RSF1010
684
EcoR1
Denaturation and Renaturation
684 insertion
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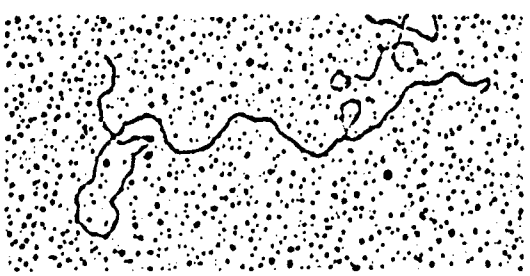

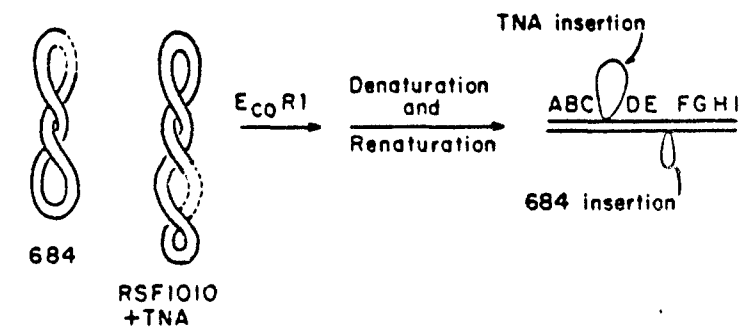
684
RSF1010 +TNA
EcoR1
Denaturation and Renaturation
TNA insertion
A B C D E F G H I
684 insertion

Figure 2. The Determination of the Polarity of TnA Insertion into Plasmids.

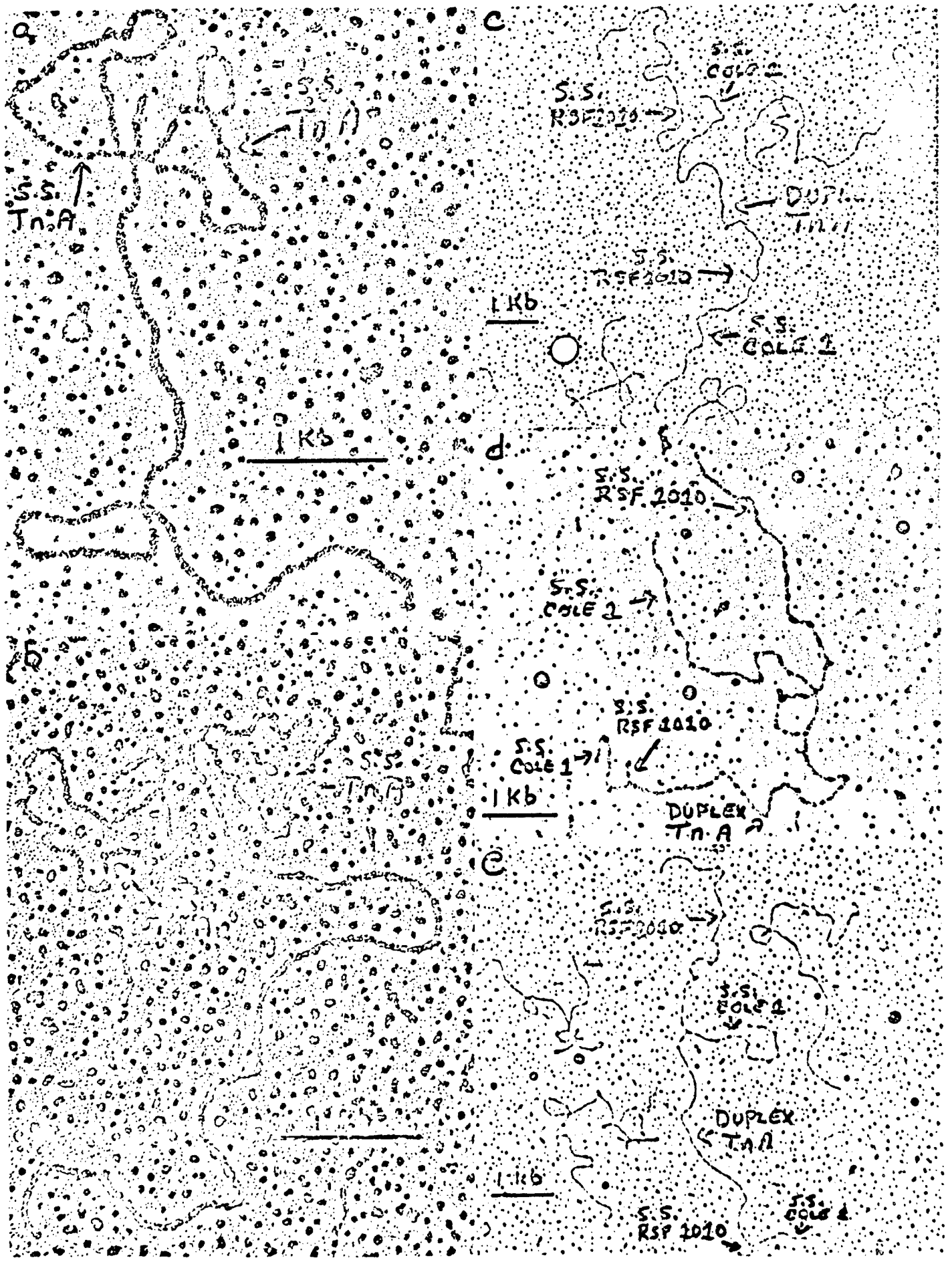
a
S.S.
Tn.A
1 Kb
b
S.S.
Tn.A
1 Kb
c
S.S.
RSF 2010
S.S.
COLE 1
DUPLEX
Tn.A
1 Kb
d
S.S.
RSF 2010
S.S.
COLE 1
S.S.
RSF 1010
DUPLEX
Tn.A
e
S.S.
RSF 2010
S.S.
COLE 1
DUPLEX
Tn.A
1 Kb
S.S.
RSF 1010
S.S.
COLE 1

TABLE 1

PHENOTYPES OF RECOMBINANTS ISOLATED FROM TRANSFORMING *E. COLI* C600 WITH PLASMID DNA ISOLATED FROM REC^+ OR REC^- HOST

DNA From	Total Colonies	A^+, S^+, Su^+ GROUP I	A^+, S^-, Su^+ GROUP II	A^+, S^-, Su^- GROUP III	A^+, S^+, Su^- GROUP IV	A^+, S^+, Su^+, Cm^+ (Rldrd)[a]
REC^-	200	173	15	3	6	3
REC^+	200	166	25	4	5	0

[a]Plasmid DNA characterized to be Rldrd DNA and not another recombinant.

TABLE 2

MINIMAL INHIBITORY CONCENTRATION (MIC)
OF VARIOUS PHENOTYPIC GROUPS ACQUIRING TnA

	Plasmid	Site of Insertion From Left Hand End	Phenotypic Class	Antibiotic Resistance: Ampicillin	Streptomycin	Sulfonamide
	B1M4	94.6%	IV	3 mg/ml	10 ug/ml	< 10 ug/ml
	B2M7	96.7%	IV	3 mg/ml	20 ug/ml	< 10 ug/ml
	B2M5	98.1%	IV	3 mg/ml	35 ug/ml	< 10 ug/ml
	A1M5	94.6%	III	3 mg/ml	5 ug/ml	< 10 ug/ml
	R-M8	2.9%	II	3 mg/ml	1 ug/ml	< 10 mg/ml
	R-M5	4.19	II	3 mg/ml	1 ug/ml	< 10 mg/ml
	I-M3	3.36	II	3 mg/ml	1 ug/ml	< 10 mg/ml
	R-M4	45.2	I	3 mg/ml	60 ug/ml	< 10 mg/ml
	B2M-3	42.1	I	5 mg/ml	160 ug/ml	> 10 mg/ml
	B2M-4	24.6	I	3 mg/ml	60 ug/ml	< 10 mg/ml
(Sensitive)	C600	—	—	10 ug/ml	< 1 ug/ml	< 10 ug/ml
(Wild-type)	RSF1040	—	—	< 10 ug/ml	40 ug/ml	< 10 mg/ml

Table 3

EFFECT OF INSERTION OF TnA
ON COPY NUMBER

Strain	Plasmid	Site of Insertion From Left Hand Ent Of Molecule	Plasmid Copies Per Chromosome[a]	% Total Plasmid DNA
HB101	RSF1010	-	25	5
	B_2M_2	1.8%	26	9
	B_2M_3	42.1%	45	15
C600	A1M5	95.0%	17	6
	R-M4	45.0%	17	6
	R-M1	42.0%	27	9
	RSF1010	-	17	6

[a]Number of copies of plasmid DNA per chromosome equivalent was calculated according to the formula:

$$\frac{\text{total plasmid CPM}}{\text{total chrom. CPM}} \times \frac{\text{Chrom. MW } (2.5 \times 10^9 \text{ dalton})}{\text{Plasmid M.W.}}$$

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The antibiotic resistance gene of R plasmids which encodes for ampicillin resistance resides upon a 3.7×10^6 dalton sequence of DNA, TnA, which can migrate (translocate) from plasmid to plasmid. The translocation of TnA occurs independently of normal bacterial rec functions so that it permits rearrangement and recombination of DNA species from diverse backgrounds. The insertion of TnA is mutagenic and may have either a polar effect or a promotor effect depending upon its orientation of insertion. Translocation sequences such as

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TnA are powerful tools for genetic research since insertion of a resistance gene into a DNA molecule provides a readily identifiable genetic and physical marker. This feature has been exploited to mark Ent and K plasmids and can be used for the construction of vaccine strains. The epidemiological implications of the discovery that antibiotic resistance genes can migrate from molecule to molecule are most significant. No longer should one simply think of the direct extension of drug resistance plasmids by transfer but rather of an enormous reservoir of genes available to insert into any DNA species.