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CONJUGATIVE TRANSFER IN THE DISSEMINATION OF BETA-LACTAM AND AMINOGLYCOSIDE RESISTANCE

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1. ABSTRACT

The dissemination of antibiotic resistance among pathogenic bacteria can be attributed largely to conjugative DNA transfer. The general category of conjugative transfer includes both bacterial plasmid conjugation and the transfer of nonreplicative conjugative transposons. Prototypes for these two systems are the plasmid RK2 and the conjugative transposon Tn*916*. To address the long-term problem of the increasing prevalence and severity of antibiotic resistance, strategies aimed against conjugative transfer are needed, but their development will require a greater understanding of conjugative resistance gene acquisition. Overviews of the two conjugative transfer systems are presented, to summarize and compare current concepts. Observations regarding transfer of conjugative transposons is consistent with the prevailing model for plasmid conjugation, that is, by the transfer of a single-stranded DNA molecule from the donor to the recipient bacterium, and the generation of the single strand by rolling circle DNA replication. The relevance of vegetative plasmid replication and host range to the spread of multiple drug resistance is discussed, and clinical examples of conjugative transfer of multiple antibiotic resistance illustrate the severity of the current situation. Possible directions, traditional and innovative, are offered to address the conjugative transfer problem in drug resistance, and potentially to break the cycle of antibiotic development followed by the bacterial resistance gene acquisition.

2. INTRODUCTION: THE CYCLE OF RESISTANCE

The cycle of the discovery and development of new antibiotics, followed by the bacterial response of new antibiotic resistance, began in the 1950s and continues unabated. The phenomenon of the cycle of resistance documented over the past 40 years has prominently included the dissemination of genes for resistance to the β-lactams and the aminoglycosides. A recently written history of the evolution of β-lactam resistance aptly illustrates the cycle. What began as two forms of penicillin, penicillin G and penicillin V, rapidly brought forth a family tree of semisynthetic penicillins, made necessary by successive rounds of new resistance (1). This chapter on the conjugative transfer of antibiotic resistance genes derives significance from the following generalizations that can be made regarding the dynamics of the cycle of resistance (figure 1). The critical determinants in the cycle are the rates of the emergence of each new resistance genotype and of the development of each new antimicrobial agent, that is, the time required for the completion of one cycle. The rates then determine the lifespan of usefulness of a given antimicrobial agent, developed with considerable outlay of intellectual and financial investment. The cycling rate for a given organism and given antimicrobial agent is a function of two main factors: the mode of acquisition of the resistance genotype and the strength of the selective pressures for the new resistance genes for bacterial survival.

The pressures selecting resistant organisms are often immense, and result from the increasing worldwide use of antibiotics for medical and nonmedical purposes. Furthermore, the clinical treatment of bacterial infection often impacts both the targeted pathogenic organisms and the resident microflora. Resistant species among the normal microflora have thereby provided resistance gene pools for later dissemination to pathogenic bacteria. The causes of the resistance phenotype are sometimes multifactorial and dependent on multiple gene products, but are more often attributed to single genes.

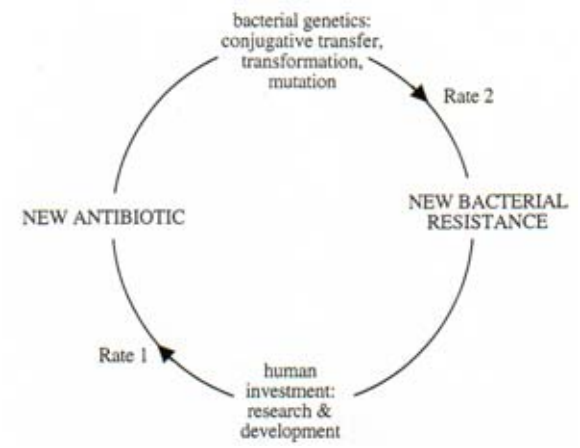

Figure 1. The cycle of resistance. Rate 1 is the rate at which the new antibiotic can be developed and Rate 2 is the rate at which bacteria develop resistance to the new antibiotic

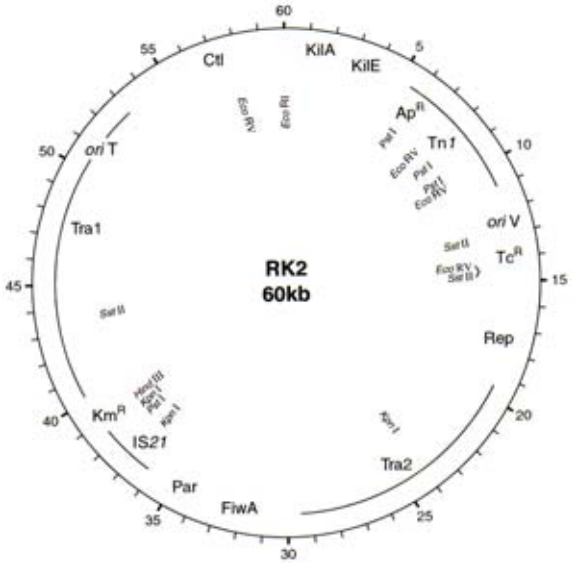

Figure 2. Map of the 60 kb IncP conjugative plasmid RK2. Conjugative transfer genes are clustered in two genetic regions, Tra1 and Tra2.The origin of transfer (*oriT*) and DNA-processing genes are part of Tra1, and the mating pair stabilization and pilus assembly genes are part of Tra2. Selected restriction enzyme sites and the antibiotic resistance genes for ampicillin (Ap^R), tetracycline (Tc^R), and kanamycin (Km^R) are shown, and other genetic regions are indicated, according to the abbreviation conventions established in reference 29.

Bacterial acquisition of resistance genes occurs by a limited number of operatives: mutation within a single gene, or the acquisition of a new gene. The latter, the acquisition of a new gene, generally occurs by genetic transformation or by conjugative transfer. The process of mutation is slower in achieving a resistance genotype by several orders of magnitude. While spontaneous mutations that yield an antibiotic-resistance bacterial colony normally occur at frequencies of 10^{-6} to 10^{-10}, per organism, resistance gene transfer can occur at a rate of 10^{-1} per organism. The expected rate of multiple antibiotic resistance by independent mutations would be the product of each rate, so for two antibiotics, the resultant rate could be 10^{-8} X 10^{-8}, or 10^{-16}. It has been suggested that mutator phenotypes emerge more often in pathogenic than in nonpathogenic populations, ten times more often in one study, but such observed rates are generally still in the 10^{-6} to 10^{-10} range (2). The acquisition of resistance by conjugation occurs at significantly higher frequency and efficiency, and several resistance genes can be acquired simultaneously, all in one event, at a rate of 10^{-1} per organism. Thus, the rate of the development of antimicrobial resistance throughout a given population is for most bacteria the greatest when the acquisition of resistance is by gene transfer. Finally, gene acquisition by transformation becomes less efficient and lower in frequency as the size of the incoming DNA increases, whereas conjugative gene transfer is highly efficient for large DNA segments.

Over time, mutations do accumulate, and gene mutation and gene transfer together contribute to the antibiotic resistance phenotype. The transfer of a β-lactamase and the mutation of penicillin binding proteins have together contributed to high levels of resistance to β-lactams (1), and it has been noted that mutator phenotypes may emerge more often in pathogenic strains (2). However, conjugative transfer has been of the greater consequence in the rapid dissemination of multiple resistance to such antibiotics as the β-lactams and aminoglycosides among most clinically important organisms. The result may be simply the continuation of the cycle of resistance, but more significantly, it may be the interruption of the cycle as the bacterial resistance rates outpace the rates of new agent development. Such a predicament is illustrated by horrific examples of untreatable and fatal infections such as the neonatal septicemia mediated by the multiply resistant *Klebsiella pneumoniae* EK105 (3).

Gene transfer occurs by bacterial conjugation as mediated by plasmids or by the conjugative transfer systems of conjugative transposons. Plasmid-mediated bacterial conjugation can result in self-transfer of a large plasmid and/or the mobilization of smaller plasmids. Conjugation is illustrated experimentally in table 1. In these mating experiments, the entire transfer system is encoded on the large plasmid RK2 (figure 2), and the smaller plasmid, pUC(Cm)$oriT_{RK2}$, requires for mobilization only the sequence called the origin of transfer (*oriT*). The *E. coli*-to-*E. coli* conjugation results are expressed as frequencies of plasmid transfer to the recipient cells, and they demonstrate the efficiency and absolute dependence on RK2-encoded functions for both self-transfer and for mobilization of the small plasmid. The *oriT* sequence, required *in cis,* is processed for DNA transfer and includes the origin and the terminus of the transferred DNA strand. Gene transfer by conjugative transposition occurs by a conjugative apparatus encoded by a large genetic element. Displaying some of the attributes of transposons, these conjugative elements are incapable of independent replication in either the donor or the recipient

Table 1. *E. coli* to *E. coli* RK2-mediated Conjugation*

Donor (2×10^7)	Recipient (8×10^7)	Transconjugants	Transfer Frequency (transconjugants per recipient)
DH5 pUC(Cm^R)	HB101(Sm^R)	HB101 pUC(Cm^R)	
		None	$< 8 \times 10^{-7}$
DH5 pUC(Cm^R)oriT	HB101(Sm^R)	HB101 pUC(Cm^R)oriT	
		None	$< 8 \times 10^{-7}$
DH5 pUC(Cm^R)	HB101(Sm^R)	HB101 pUC(Cm^R)	
RK2 (Km^RTc^R)		None	$< 8 \times 10^{-7}$
		HB101 RK2(Km^RTc^R)	
		4×10^7	0.50
DH5 pUC(Cm^R)oriT	HB101(Sm^R)	HB101 pUC(Cm^R)oriT	
RK2 (Km^RTc^R)		6×10^7	0.75
		HB101 RK2(Km^RTc^R)	
		4×10^7	0.50

*Values given are the numbers of bacteria per milliliter which grew under selection by the appropriate antibiotics after a one hour conjugation experiment. Markers selecting for the donor bacteria were the plasmids markers choramphenicol (Cm^R), kanamycin (Km^R), and tetracycline (Tc^R), and, for the recipient bacteria, streptomycin (Sm^R) was the chromosomally-encoded resistance marker. For transconjugants, which are recipients which have received plasmid(s) from donors, the markers were streptomycin and the particular plasmid marker. In conjugation experiments in which no transconjugants appeared on the selective plates, even without prior dilution of the mating mixture, the absence of growth of transconjugants is indicated as "none."

cell. The maintenance of conjugative transposons in bacterial populations therefore depends on rapid integration into the recipient bacterial genome after transfer.

While the role of conjugative gene transfer has been widely recognized as the pre-eminent genetic route towards antimicrobial resistance in many organisms, especially in the simultaneous acquisition of several resistance genes, the subject often gets perfunctory treatment in review articles. This is because conjugative gene transfer *per se* has not been considered a target in the development of new strategies to combat the antimicrobial resistance cycle. Why is this the case? The answer stems from the fact that our understanding of the basic science of gene transfer by conjugation has been insufficient to provide the concepts for short-term or long-term strategies. Although considerable progress has been achieved over the years, the slowly evolving models have yet to offer innovative applications that address the problem of drug resistance transfer. What is needed is greater insight into the biochemical events of conjugative gene transfer, to identify molecular activities that are unique and can that then be targeted. Inhibitors that are analogous in utility to the β-lactamase inhibitors could be designed to work against conjugation, towards an *in vivo* containment of resistance genes (1). Such inhibitors could be part of more complex clinical treatment regimes. The result could be the slow-down in the rate of spread of bacterial resistance. At this time, there are no such transfer inhibitors, and the development of these strategies may require years. However, treatment is becoming more customized to the organism, a trend that may accommodate the addition of agents designed to target resistance gene transfer.

In recent years the pace of development of new antimicrobials has slowed. This can be attributed to the gradual exhaustion of resources, resources such as the soil antimicrobials and their semi-synthetic derivatives. Continued antimicrobial treatment will surely keep up the selective pressures, and the resistance problem is expected to become more severe, not less. The issue is more complex in the two-thirds world, where cost containment is essential. Lastly, appropriate antimicrobial treatment has recently become a challenge of greater significance as bacteria are attributed for the first time as agents of diseases such as gastric ulcers and carcinoma, kidney stones, cardiovascular disease, and most recently, Alzheimer's disease (4, 5, 6,7).

3. CONJUGATIVE TRANSFER

For the purposes of this review, conjugative transfer will include the two major conjugative phenomena responsible for the spread of antimicrobial resistance genes among bacteria: plasmid conjugation and the transfer of conjugative transposons. Bacterial conjugation and conjugative transposition are found among both Gram-negative and Gram-positive organisms. Genetic relatedness of transfer functions has been found among some of the different incompatibility (Inc) groups of conjugative plasmids and between conjugative plasmids and conjugative transposons (8, 9). One unifying theme for this discussion is that bacterial conjugative systems have basic properties that appear to be recipient-independent. Conventional transposition, considered here as auxiliary and involving other transfer mechanisms to disseminate genes among bacteria, will not be discussed, nor will the two other significant modes of resistance gene acquisition, transformation and mutation.

Common mechanisms of antibiotic resistance gene acquisition among clinical organisms are shown in table 2. This perusal is intended to demonstrate the prominent role of conjugative transfer in disseminating antibiotic resistance among diverse bacterial pathogens. While it is true that the earlier and more rapid spread of plasmid-mediated drug resistance occurred among the Gram-negative bacteria, the slower emerging resistance problems among Gram-positive

Table 2. Genetic mechanisms of antibiotic resistance acquisition among common pathogenic bacteria[1]

Mutation	Natural Transformation	Conjugative Transfer (Plasmid and Conjugative Transposon)
❖ *all bacteria* ❖ *M.tuberculosis*[2]	❖ *Acinetobacter* ❖ *Enterococcus* ❖ *Helicobacter* ❖ *Haemophilus* ❖ *Neisseria* ❖ *Staphylococcus* ❖ *Streptococcus*	❖ *Enterobacteriaceae* ❑ *Enterobacter* ❑ *Escherichia coli* ❑ *Klebsiella* ❑ *Proteus* ❑ *Salmonella* ❑ *Shigella* ❑ *Serratia* ❖ *Acinetobacter* ❖ *Bacteroides* ❖ *Campylobacter* ❖ *Clostridia* ❖ *Enterococcus* ❖ *Haemophilus* ❖ *Helicobacter* ❖ *Listeria* ❖ *Mycoplasma* ❖ *Neisseria* ❖ *Pseudomonas* ❖ *Staphylococcus* ❖ *Streptococcus* ❖ *Vibrio* ❖ *Yersinia*

1.Compiled from references 10-20. 2.Acquisition of resistance in *M. tuberculosis* has thus far been attributed exclusively to mutation. Gene transfer in *M. tuberculosis* by transduction and transformation has been established, but not in antibiotic resistance acquisition (21).

organisms is also associated with conjugative gene transfer. The mutation-driven drug resistance of the pathogen *Mycobacterium tuberculosis* is an exception to the rule (21,22). This organism is also unusual among both slow-growing and fast-growing mycobacteria in that no plasmids have yet been found to be carried by *M. tuberculosis* strains (23), and plasmid-mediated conjugative gene transfer has not been demonstrated in *M. tuberculosis* as it has been in the fast-growing mycobacteria (24).

Plasmids may be integrated into the bacterial genome, by recombination, so that encoded resistance genes may be maintained over time if the plasmid cannot replicate independently within that cell. In addition, antibiotic resistance genes encoded by conjugative plasmids are often found on conventional transposons, so the drug resistance genes can be readily transposed into the bacterial chromosome or other plasmids. Thus, the resistance gene can be permanently maintained even if the incoming plasmid cannot replicate. Another mechanism of long-term maintenance is the recombination of resistance genes into integrons which may be carried by the chromosome or a resident plasmid. The potential for spread of the resistance genes by conjugative transfer may therefore ultimately involve several processes: plasmid conjugation, conventional transposition, recombination, and conjugative transposition. Indeed, integrated conjugative plasmids may not be recognized until transferred into a bacterial species which supports its replication. Such plasmids are, for a time, functionally analogous to the nonreplicative conjugative transposons. An example of this type of scenario was recently reported for *Haemophilus influenzae* (25).

3.1. Bacterial Conjugation

Conjugation is a process by which DNA is transferred from one bacterium to another via cell-cell contact and energy-driven transport. The process is conceptualized as two sub-processes: DNA preparation, and mating bridge formation. DNA preparation includes the process of relaxosome formation. A relaxosome is the DNA/protein complex created by the binding of certain transfer proteins to regions within the origin of transfer (oriT). This complex effects a single-stranded nick in the DNA at the nic site, and the DNA is topologically "relaxed" upon denaturation of proteins. It is the single-stranded DNA generated by the nick that is thought to be unwound and transferred. The mating pair, the donor and recipient bacterial cells, is thought to be stabilized by the conjugative pili. The mating bridge connects the cells as the DNA is transferred, so that, unlike bacterial transformation, conjugation is unaffected by the presence of DnaseI. The mating bridge is a complex multi-protein apparatus of largely unknown structure. DNA transfer by bacterial conjugation is mediated by a large plasmid which encodes the transfer functions. The conjugative plasmid may replicate independently within the bacterial cell, or be integrated into the bacterial genome.

There are many plasmid incompatibility (Inc) groups known to be conjugative, falling into loosely-defined groups based on genetic relatedness and pilus structure. One

group is comprised of the IncF-like conjugative plasmids, including IncF, IncS, IncC, IncD, and IncJ plasmids. The second group consists of the IncP-like conjugative plasmids, including IncP, IncU, IncM, IncQ, IncW. A third group, with similarities to the IncP plasmid group but with a different pilus structure, could be called the Ti plasmid group and includes IncX, IncH, IncN and IncT (26). A fourth group, the IncI group, also of genetic similarity to IncP plasmids but with unique pilus properties, consists of IncI, IncB, and IncK (27). Conjugative plasmids in the first two groups appear to account for most of the drug resistance gene transfer among clinical isolates. The 100 kb F (IncF) plasmid (28), and the 60 kb RK2 (IncP) plasmid (29) are the first conjugative plasmids to be entirely sequenced, and they have been the prototypic plasmid systems for Gram-negative bacteria. Studies of F and RK2 have led to the following model to describe bacterial conjugation:

1. donor and the recipient cell contact and mating bridge formation,
2. DNA relaxosome formation initiated by a single-stranded nick within the *oriT*,
3. conjugative "rolling circle" replication and single-stranded DNA transfer to the recipient,
4. DNA recircularization, complementary strand synthesis, and vegetative replication in the recipient.

These four categories of events will be described as an overview, based on similarities and differences between the IncF and IncP plasmid conjugative systems. The reader is referred to reviews that describe in greater detail the functions and putative functions for the numerous gene products of these plasmid transfer systems (28-31).

3.1.1. Donor bacterium and recipient cell contact and mating bridge formation

Cell contact is facilitated by conjugative pili, which promote donor and recipient cell interaction and stable mating pair formation. These pili are encoded by transfer genes of the conjugative plasmid carried by the donor bacterium. In the case of the IncP plasmid RK2 (identical to RP1, RP4, and R68), mutant studies have established that extended pili are not an absolute necessity for bacterial conjugation, although several pilus-assembly-related gene products are required (30). In the Ti (tumor-inducing) plasmid of *Agrobacterium tumefaciens* and in the F-like plasmid systems, pili are considered essential for conjugative DNA transfer. However, the distinction between extended pili and basal pilus structures has not been genetically and functionally explored with these other plasmid systems. In F, pilus retraction is believed to establish mating pairs. Such a role for conjugative pili is no longer postulated as essential for RK2 conjugation, although the conjugative bridge is thought to involve pilus components. A complex, multi-component structural model for the mating bridge has not been worked out for either system, and such models may be key in understanding the most important differences between the IncF, IncP, and other plasmid groups.

oriT$_{RK2}$-containing mobilizable plasmids have been studied in regard to the mating bridge in *E. coli* (32). By Southern blot hybridization using an *oriT* sequence probe, the *oriT* has been localized to the inner membrane and not to the outer membrane (33). Similar experiments have been done using radiolabelled RK2 *oriV* sequences as the probe, and the *oriV* was likewise found within inner membrane fractions of cell extracts (34). These results suggest that the RK2 plasmid, with 5 to 8 copies per chromosome at 37^{o}C, may be attached to the inner membrane throughout much of the cell cycle. This is consistent with ideas regarding partitioning of plasmid molecules during cell division by attachment to Par proteins. During conjugation, when cell-cell contact is made and the membrane pore structure is assembled, plasmid attachment to the cytosolic side of the inner membrane would provide plasmid DNA access to a conjugative bridge structure that spans the membranes. The nicked form of the DNA could then readily initiate donor-to-recipient transfer.

Consistent with these observations are the results obtained by permeability studies. When mating bridge genes are cloned into *E. coli*, there is an increase in cytoplasmic membrane permeability to inorganic and organic ions, and to ATP, and this increase in permeability can be reversed by the addition of the relaxosome (35). It therefore appears that the relaxosome resides at the base of the mating bridge transfer structure. The two events, relaxosome formation and mating bridge formation, may be jointly controlled by a mating signal initiated by donor-recipient cell contact. Transport of the DNA strand through the membranes, for Gram-negative bacteria, requires a pore to be formed that traverses the inner and outer membranes of both the donor and recipient cells. With no outer membrane, the Gram-positive bacteria might not require a diversity of components for the conjugative bridge. DNA transfer among Gram-positive bacteria has indeed been thought to be a simpler process, involving a genetic unit of about half the size of the unit for Gram-negative conjugative systems, but the difference may be due in part to the conjugative pili production of Gram-negative bacterial systems (36).

Over recent years it has become more widely held that the conjugative mating bridge is a donor-dependent structure that does not require a recipient receptor-like molecule. This concept is supported by conjugation experiments using mobilizable shuttle vectors that have replication regions customized for very diverse recipient cells. Given the appropriate replicon for replication within the recipient, conjugative proficiency appears to be unlimited in host range. Conjugation is feasible, for example, from bacteria to diverse species of other bacteria, to yeast, to plant cells, and, most recently, to mammalian tissue culture cells (37-41). What varies from recipient to recipient is the efficiency of the conjugative transfer event, that is, the frequency of one event per time period per donor or recipient cell. Frequency values for divergent donor and recipient bacterial cells are typically low, perhaps due to a low probability or stability of mating pair formation under the conditions used. Very high frequencies are obtained from *E. coli* to *E. coli* on solid media using the RK2 plasmid system (table 1). High frequencies are also obtained with *E. coli* using the F

Table 3. Redefining Host Range*

Donor	Recipient	Plasmid System	Frequency of Transfer
E. coli	*E. coli*	RK2	10^{-1}
E. coli	*E. coli*	F	10^{-1}
E. coli	*Pseudomonas aeruginosa*	RK2	10^{-1}
E. coli	*Pseudomonas aeruginosa*	F	10^{-5}
E. coli	*Saccharomyces cerevisiae*	RK2	$10^{-5} – 10^{-7}$
E. coli	*Saccharomyces cerevisiae*	F	10^{-7}

*Conjugation experiments to *E. coli* and *Pseudomonas aeruginosa* to compare the F and RK2 plasmid systems were done by constructing a chimeric shuttle vector carrying transfer origins of both plasmid F and RK2 systems, and mobilizing with either F or RK2. Data summarized from references 39, 44, and 45.

plasmid system, given the enhanced cell-cell contact provided by the F pilus adhesin (42). The important distinction to be made here is the difference between feasibility and frequency: essential components, which are the minimum requirements, must first be defined in quantitative studies, and auxiliary components, which are the frequency-enhancing factors, can then be identified for a given system. Low frequency events must be distinguished from nonconjugative acquisition of marker resistance, such as by low frequency spontaneous mutation. Among conjugation systems that are known to be regulated, such as the F and Ti systems, derepression of genes is needed to optimize transfer frequency. In the F plasmid system, pilus recognition of a specific recipient receptor has been thought to initiate mating-pair formation (42), but recently such receptors have been considered unessential. Pilus/recipient recognition may in fact increase transfer frequency, as do a number of other non-essential conjugation components which appear to be dispensable under certain conditions. The fact that both the F and RK2 plasmid systems have mediated transfer to yeast cells, and at comparably low frequencies, suggests that extended pili have analogous functions in the two systems, that is, enhancing mating pair formation for bacteria, which would increase conjugation frequency. Hence, for RK2, a subset of conjugative genes which have been called essential and required for "mating pair formation," might be more precisely defined as the genes for "mating bridge formation."

The low frequency transfer events from *E. coli* to yeast, experimentally documented with the RK2 and the F plasmids, should also be instructive in discerning the role conjugative plasmids play in disseminating antibiotic resistance genes (39). Historically, the IncF prototype F plasmid has been considered the definitive narrow-host-range plasmid, transmissible only among enteric bacteria, while the IncP RK2 plasmid has been considered the definitive broad-host-range plasmid, transmissible among both enteric and non-enteric bacteria at high frequency. Defining host range originally included the abilities to transfer and to replicate within the recipient after transfer. As the field became more sophisticated, particularly with the advent of shuttle vectors designed for replication in the donor bacteria and in diverse conjugation recipients, the conjugative host range could be distinguished from replicative host range. RK2 always appeared to have broader conjugative host range than F, but early judgments may have been based more on transfer frequencies and replicative host range than on the conjugative host range. RK2 plasmid replication has a broader bacterial host range than F plasmid replication, but even RK2 requires engineered recipient-specific replicons for bacterial recipients that do not support RK2 replication, such as *Bacteroides* (43). Both F and RK2 conjugate more efficiently from *E. coli* to bacteria than to yeast. Both F and RK2 appear to have a somewhat generic transfer apparatus which facilitates conjugation to related and to highly unrelated organisms, although the conjugative system of RK2 has an overall higher efficiency and is the system of choice for shuttle vector construction (table 3). Factors enhancing conjugation efficiency presumably include the quality of mating pair formation and of the mating bridge.

Redefining host range is in fact relevant to a discussion of the dissemination of antibiotic resistance among bacteria. We have described how both narrow host range and broad host range conjugative plasmids could conceivably mediate transfer of resistance to divergent species in clinical situations. Thus, the "narrow host range" plasmids are potentially "broad host range" but transfer to certain bacteria at lower frequency. The transfer frequencies vary according to the bacterial species acting as recipient, the conditions in which the mating pairs are established, the existence of auxiliary replicons on the plasmid, and unknown factors which may foster conjugation in natural environments such as the mammalian host. In the dissemination of antibiotic resistance, the resistance gene is often carried on genetically mobile elements, as discussed above, so the replication of the conjugative plasmid within the new recipient may not be necessary to secure the residence of the gene within the bacterium. Furthermore, a very low frequency event may be entirely sufficient under conditions of strong selective pressure. An IncF plasmid may encode virulence determinants, some of which are known to enhance survival in the host, and antimicrobial resistance determinants (46). Therefore, for this plasmid group, selective pressures can be multiple and ultimately overcome host range and transfer frequency limitations.

The process of heterologous relaxosome mobilization is an interesting phenomenon that has provided insights into mating bridge formation. The IncQ plasmid RSF1010 is not self-transmissible, but can be efficiently mobilized by the IncP, IncI, IncX and Ti plasmids, and less efficiently by the IncF, IncN, and IncW plasmids (47, 48). Those trans-acting components essential for RSF1010 mobilization have been determined for RK2. A total of 12 gene products are required for mobilization: TraF, TraG, TrbB, TrbC, TrbD, TrbE, TrbF, TrbG, TrbH,

Table 4. RK2-encoded Functional Cell Surface Components*

Function	Required Components
Heterologous relaxosome mobilization	TrbB--TrbJ, TrbL, TraF, TraG
Donor-specific phage adsorption	TrbB--TrbJ, TrbL, TraF
Donor-specific phage production	TrbB--TrbJ, TrbL, TraF, TrbK
Extended pili	TrbB--TrbJ, TrbL, TraF
Surface exclusion	TrbK

*Surface-related required proteins and functions, described in the text, compiled from references 30, 49, and 50. TrbB--TrbJ indicates the RK2 gene products TrbB, TrbC, TrbD, TrbE, TrbF, TrbG, TrbH, TrbI, TrbJ, and TrbL.

TbI, TrbJ, and TrbL. (32). All 12 are believed to be associated with the bacterial cell membrane, including TrbB, a protein which does not possess the properties of a typical hydrophobic membrane protein (30, 33). The surface exclusion lipoprotein TrbK, which is required for the production of extended pili, is not required for either heterologous relaxosome mobilization or for IncP donor-specific phage adsorption (table 4). Phage adsorption is the first step in the multi-step process of phage infection and production. For donor-specific phage production, visualized as phage plaques, both TraF and TrbK and the other 10 Trb gene products listed are required, but TraG is not required (table 4). For IncP plasmids, then, the components necessary for extended pili are also needed for donor-specific phage production, but not for phage adsorption or mobilization of relaxosome DNA.

The significance of these observations includes the following. The diversity of plasmid systems capable of mobilizing RSF1010 suggests a functional conservation of a donor-specific mating bridge, with some variation within the overall conservation evidenced by differences in efficiency among the plasmids. This is consistent with RK2 transfer experiments showing a seemingly unlimited host range of cellular recipients, including yeast, plant cells, and mammalian cells. Secondly, the functionally conserved mating bridge may accommodate two-way traffic. Donor-specific phage cannot infect cells without the mating bridge, and the bridge apparatus/phage receptor is needed at the earliest stages of infection, probably at the time of adsorption and DNA injection. Thirdly, extended pili do not function in what has long been considered most important pilus-mediated functions: phage adsorption and DNA transfer. These observations necessarily change our approach to model-building.

With the re-evaluation of previously-held views that the donor-specific phage adsorbed to pili and that pili were directly involved in DNA transfer, how can the roles of pili and pilus-assembly components now be functionally distinguished? By electron microscopy, IncP pili are seen in bundles, and often appear to be shed from the bacterial surface (32). These observations suggest a new possibility for the mating bridge, comprised of TraF, TraG, and a basal pilus structure perhaps created after the pilus has been shed or broken off from the cell surface. Thus, RK2 pili may be shed from the surface, while F pili may be retracted by a pilus depolymerization process at the membrane. There is to date no direct evidence for either event, but the circumstantial evidence from both systems point to some sort of residual basal pilus structure that serves as the mating bridge. Phage appear to adsorb to saturation at the "mating bridge" surface receptors, involving TraF but not TraG, with up to 25 phage per *E. coli* cell and 60 per *Salmonella typhimurium* cell (35, 51). Only TrbK is needed to mediate surface exclusion, to greatly reduce the frequency of a donor cell acting as a recipient (table 4). These studies support a model in which components such as TrbK and TraF have more than one function. Further studies will be of interest in clarifying the activities of these and other mating bridge components in RK2 conjugation and in donor-specific phage infection.

To help dissect the transfer process, conjugation has been separated into two functional parts, DNA preparation, and mating bridge formation. The connecting link between DNA preparation and the mating bridge may be supplied by the two RK2 proteins TraF and TraG, and their analogs in other systems. In RK2, these two proteins appear to be co-transcribed and co-translated (49, 52). The TraG protein has an NTP-binding domain and might thereby provide energy for DNA transport, working together with TraF which has sequences suggesting a role as a DNA processivity factor (53, 54). Both TraF and TraG are absolutely essential for conjugation (49). The relaxosome may begin transfer by interacting with TraF and TraG, which are either closely associated with, or are part of, the mating bridge. The *traG* gene appears so far to be well-conserved throughout the conjugative plasmid world, with homologs in the Ti, F, R100, R388, CloDF13 plasmids, and a more distant homolog in the Gram-positive plasmid pGO1 (47, 53). In cross-complementation studies, TraG of RK2 actually increases the mobilization frequency of RSF1010 by the *traG*-minus mutant of IncW plasmid R388, relative to the mobilization frequency of native R388 (47). The *traF* gene is not as highly conserved among conjugative plasmids, with homologs found only among more related conjugative plasmids such as the IncPβ R751 plasmid and the Ti plasmids of *Agrobacterium tumefaciens* (55). However, the role of TraF may be supplied by nonhomologous and uncharacterized transfer functions.

Recently, it has been shown that TraF has a unique signal peptidase activity which processes the proposed prepilin subunit TrbC, but not those proteins that are processed by the *E. coli* signal peptidase Lep (55). Antibody studies have shown that TraF and TraG of RK2 can be localized to both the inner and outer membrane

fractions of *E. coli* cells, and alkaline phosphatase fusions generated by Tn *phoA* insertion suggest that TraF is more surface-exposed than is TraG (33). The RK2 transfer membrane structure, comprised of TraF, TraG, and the basal pilus components, may therefore bring together inner and outer membranes as part of the mating bridge, with TraF more external than TraG. The use of these localized regions of fused inner and outer membranes may simplify the mating bridge building process for Gram-negative bacteria. TraF is required in some specialized way for incoming phage DNA, while TraF and TraG together, possibly in equimolar amounts, are required for the exiting of conjugating DNA from the donor cell (table 4). Complementation of mutant RK2 donors (*traF* or *traG*) can be accomplished by the corresponding gene in the donor and not in the recipient (33, 49).The concept of membrane fusion sites at the transfer bridge, which may also serve as phage adsorption sites, is reminiscent of Bayer's fusion sites. Bayer observed that some T phage appeared to adsorb at such membrane adhesion sites, visualized by electron microscopy as points of contact between the inner and outer membranes of plasmolyzed *E. coli* cells (56). If such sites are part of the conjugative transfer or phage infection process, the question could be asked whether the transfer apparatus induces the putative membrane adhesion sites or capitalizes on pre-existing, perhaps transitory, sites.

Taken together, the various experimental approaches described here suggest that the conjugative bridge is a multi-protein apparatus spanning the cytoplasmic membrane, and, in the Gram-negative organisms, the outer membrane. The relaxosome is at the base of the bridge, poised for conjugative DNA transfer. This positioning of the relaxosome serves to decrease the permeability that is inherently associated with the presence of the mating bridge. The signal for transfer, probably produced by cell-cell contact and involving pilus components, leads to the opening of a donor-to-recipient mating bridge pore and the rapid transfer of a nicked and protein-bound single-stranded DNA.

3.1.2. DNA relaxosome formation initiated by a single-stranded nick within the *oriT*.

The relaxosome is the DNA-protein complex that consists of the conjugative plasmid DNA and certain transfer proteins that specifically recognize and interact with sequences within the transfer origin (*oriT*). The protein complex mediates a single-stranded nick at the *oriT* DNA and maintains its characteristic relaxosome topology. The TraI relaxase enzyme converts the supercoiled plasmid DNA to a nucleoprotein complex which can become a relaxed, open circular DNA molecule upon protein denaturation. The relaxase is a transferase which becomes covalently bound to the 5' end by formation of a phosphodiester bond between the 5' phosphoryl group of the DNA and the hydroxyl of the TraI tyrosine at position 22 of the protein (57). The molecular events of relaxosome formation that prepare the DNA for transfer are sequence-specific and absolutely required for conjugation (58). Mutations that abolish relaxosome formation also abolish conjugative transfer (58). In order to mobilize a small plasmid carrying the *cis*-acting *oriT*, the mobilizing large plasmid must carry cognate enzymatic proteins specific for that *oriT* sequence. Even closely related plasmids, such as the IncPα RK2 and the IncPβ R751 plasmids, encode enzymes that do not recognize and nick within the *oriT* of the heterologous plasmid (37). However, a small *oriT*-carrying plasmid which encodes its own relaxosome-forming enzymes can be mobilized by heterologous plasmids.

In RK2, proteins essential for relaxosome formation are TraI, TraJ, and TraK (56). (TraK should not be confused with the previously discussed TrbK.) The minimal *oriT* of RK2 consists of approximately 250 bp in which the nick site is centrally located (58). The nick region is a short 8 bp sequence within the *oriT* between a 40 bp inverted repeat and the nick site. The nick region sequence is recognized by the nicking enzyme relaxase, TraI. The RK2 nick region was the first to be characterized and was defined by mutation analysis: the entire 250 bp *oriT* was randomly mutagenized, subcloned, and screened for loss of transferability *in vivo*. All mutations which abolished transfer were found to be within this 8 bp region (58).Site-directed mutagenesis identified a core of 6 to 7 of the 8 nucleotides as the most essential for DNA recognition and nicking *in vitro*, as single base pair changes within this region abolished nicking (57, 58). Although the nick region is now more precisely defined as these 6 to 7 nucleotides, the conservation of sequences often extends further into the flanking regions in both directions (table 5).

There continues to be confusion regarding the differences between the nick region and the *oriT*. It is well-established that the *oriT* is the entire sequence required *in cis* for relaxation and mobilization of a plasmid by the relaxase transfer gene products, which in RK2 are TraI, TraJ, and TraK. These proteins may be supplied *in trans*.The precise minimal *oriT* is not always rigorously defined, but the typical *oriT* occupies more than 100 bp. After the RK2 nick region was defined and found to be required for the nicking reaction *in vivo* and *in vitro*, the concept was fully embraced and many putative nick regions sequences have been aligned for comparison (60-62). Nick regions are short sequences of about 10 bp. On occasion, however, the nick region has been mistakenly called the *oriT*. The *oriT* provides extensive DNA regions for recognition and binding by relaxation complex proteins. In RK2, the TraI, TraJ, and TraK proteins interact with such extended DNA regions, facilitating TraJ and TraK binding, and TraI recognition of the short nick region and cleavage at the nick site. TraK binds to an extended region on the other side of the nick site, relative to TraJ, to wrap the DNA and form a nucleosome-like structure (63). TraJ protein recognizes one arm of the inverted repeat while it interacts with the TraI protein to align TraI for nick region recognition. The entire sequence of events provides for the efficiency and specificity of the single-stranded nick by TraI, and the covalent attachment of TraI to the 5' end that leads the DNA from donor to recipient (30).

Table 5. A Sampling of Nick Regions and Flanking DNA*

IncP Conjugative Plasmid Family	
❑ RK2	C T T C A C C T A T C C T G ↓ C C C G G C
❑ R751	C T T C A C A C A T C C T G ↓ C C C G C C
❑ pTiC58(LB)	C A C A A T A T A A C C T G ↓ C C A C C A
❑ pTF-FC2	C A A C G G T C A T C C T G ↓ T A T T G C
❑ R64	A A T T G C A C A T C C T G ↓ T C C C G T
RC Replication Plasmid Family	
❑ pC194	T C T T T C T T A T C T T G ↓ A T A A T A
❑ pUB110	T C T T T C T T A T C T T G ↓ A T A C A T
IncF Conjugative Plasmid Family	
❑ F	T T T G C G T G G G G T G T ↓ G G T G C T
❑ R46	G C T G C G T T A G G T G T ↓ A T A G C A
❑ p307	T T T G C G T A G G G T G T ↓ G G T G C T
❑ R100	T T T G C G T A G T G T G T ↓ G G T G C T

*Experimental evidence for a nick region was provided for the IncP plasmid RK2 (58); nick regions for the other systems are inferred by sequence homologies (59, 60, 61, 62, 64, 65, 66). Nick regions are underlined and the nick sites are indicated by arrows. pTiC58 is a Ti (tumor-inducing) plasmid from *Agrobacterium tumefaciens*; LB refers to the left border of the sequence transferred into the plant.

The F nick region has hot been fully characterized experimentally and there are no sequences within the F *oriT* homologous to nick regions of the RK2 family (table 5). Among IncF plasmids, there are short DNA segments upstream from the nick site which binds the *E. coli* integrative host factor (IHF). This region overlaps an imperfect inverted repeat which binds a transfer protein called TraY. The relaxase nicking enzyme for F, also called TraI, becomes covalently attached to the 5' end of the transferring strand, as in the RK2 system. The role of IHF could be functionally analogous to that of RK2's TraK in facilitating a nucleosome-like structure, and the binding of plasmid F's TraY viewed as analogous to the binding of RK2's TraJ. However, the pattern, spacing, and orientation of sequences of the two systems are quite different. The putative IncF nick region and surrounding DNA have a much different pattern than the 8 bp nick region between the nick site and inverted repeat which serves as the TraJ binding site of the IncP plasmids (58). Furthermore, the fact that more than one nick site has been reported within the *oriT* suggests that the nicking reaction is not as precise as it is in RK2 (67, 68).

How these differences between the IncP and IncF *oriT* regions might affect transfer efficiency and host range is unknown. Efficiency and host range differences probably reflect differences in both DNA processing events and in mating bridge formation. However, mobilization experiments have given more weight to the role of mating bridge components in determining efficiency and host range (10, 47). The preservation of these differences by F has presumably been important for the regulation of conjugation and phage sensitivity to promote survival in the varied habitats of enteric bacteria, from the mammalian host to the sewage system. In certain RK2 plasmids it is known that there is a similar co-regulation of conjugation frequency and phage sensitivity, although such plasmids may represent a subpopulation that is selected by phage exposure (69).

3.1.3. Conjugative "rolling circle" replication and single-stranded DNA transfer to recipient

The site-specific and strand-specific nicking at the *oriT* during relaxosome formation is very similar to the events initiating the replication of plasmids which replicate only by rolling circle replication (31, 70). There are reasons to believe that the two replication phenomena share molecular ancestry, such as the recent finding of a large plasmid carrying an integrated copy of the rolling circle replication plasmid pT181 (71). This kind of event results in a plasmid molecule with a theta replication origin and a rolling circle replication origin. With these two origins, the plasmid potentially has both types of replication. This is analogous to the situation envisioned for all conjugative plasmids, in which the *oriV* origin is the theta replication origin and the RC origin could be viewed as the conjugative replication origin *oriT*. Rolling circle (RC) plasmid replication is asymmetric, that is, the duplication of one strand is not coupled to the duplication of the other strand. What occurs in conjugation in the donor cell could then be considered analogous to the first stage of plasmid RC replication, which is the replication of the "plus" or "leading" strand starting at the double-stranded origin (figure 3). What is thought to occur in the recipient cell is likened to the replication of the "minus" or "lagging" strand, which starts at what is called the single-stranded origin in RC replicating plasmids.

Plasmids which replicate by RC replication have a Rep protein with functional similarities to the RK2 TraI protein which facilitates replication initiation and forms a covalent 5' DNA-protein linkage. Some of the Rep proteins initiating RC replication function as dimers. Plasmid gene functions for RK2 conjugation include those for relaxosome formation and for mating bridge formation, and also for a DNA primase that functions in certain recipients. RC replication initiated at the *oriT* is thus largely mediated by host proteins, since RC replication requirements consist of the conserved host bacterial DNA repair components

DNA PolIII, PolI, single-stranded binding protein (SSB), and a helicase (67). These events in the recipient probably contribute to the broad host range character of conjugation, particularly for the IncP plasmids and others that transfer a DNA-bound primase into the recipient (72, 73).

Rolling circle replication is a unidirectional replication of DNA accomplished by the extension of one strand at the 3' OH end created by the nicking event. The pairing of the complementary bases to the unnicked circular strand and the progressive displacement of the original strand gives the image of a rolling-circle with a growing tail, which is the displaced strand. This form of replication has been called sigma replication, the shape of the σ describing the appearance of the DNA in electron micrographs. This form of replication has been biochemically established for pT181, the prototypic Gram-positive rolling circle plasmid (74), and for pKYM, the prototypic Gram-negative rolling circle plasmid (75), but there is no direct evidence for rolling circle replication originating at the *oriT* of any conjugative plasmid. Inferential evidence appears to have carried the day. Supporting observations include the observed single-stranded nicking event, the delivery into the recipient of the plasmid-specific primase bound to the DNA, nick region homologies with plasmids which replicate by rolling circle replication (table 5), and the ability of incoming DNA to elude restriction-modification systems specific for double-stranded DNA.

Convincing as all these observations might be, investigators have only recently adopted the rolling circle model. It has now replaced the theta replication model for conjugation that had prevailed for 30 years, that is, until the early 1990s. The paradigm shift appears to have begun with the characterization of the nick region and the *nic* site of RK2, which strongly suggest that rolling circle replication initiates at the *oriT*. Conjugation does appear to be a replicative process that ensures plasmid maintenance in the donor and introduces a new plasmid molecule into the recipient. Over recent years the rolling circle model for plasmid replication has remained consistent with a growing body of bacterial conjugation data. Proteins attached to the transferring DNA are also transferred through the mating bridge, so the membrane pore must be large enough to accommodate a protein-bound DNA molecule. Proteins bound to the transferring RK2 DNA include TraI, the TraC primase, RecA, and the single-stranded binding protein (SSB) (30).

Conjugation of DNA and its attached proteins has recently been likened to a protein export process. It has been given its own term, unique to conjugative "export", Type V export (76), and it has also been compared to export types II, III, and IV. It is possible that there is more than one type of transport involved in conjugation, but it would seem more likely that there is a versatile mating bridge complex that can mediate transport for both conjugation and donor-specific phage infection. There may then be three distinct but interrelated transport phenomena in RK2 conjugation occurring at one cell surface site. This capability could be shared by all conjugation systems which also mediate sensitivity to phage:

1. export of pilus components during pilus assembly,
2. export of the protein-bound DNA molecule in conjugative DNA transfer to a recipient cell, and
3. import of conjugative-donor-specific phage DNA, upon infection with such phage.

The IncN, IncP, and Ti plasmid groups have mating bridge-related regions of genetic homology with the Type II export genetic regions of *Bordetella pertussis* which are dedicated to the export of the pertussis toxin (26). Genetic loci sharing homology with these regions have recently been extended to two other bacterial species: the export protein Cag of *Helicobacter pylori* (77), and the intracellular multiplication (*icm*) genes of *Legionella pneumophila* (78). What is remarkable about the *icm* gene products is that they were found to be essential for two distinctly different processes: the entry of the *Legionella* into human macrophages, which is part of the *Legionella* infection process, and for the mobilization of the plasmid RSF1010 between *Legionella* donor and recipient strains (78). Since the macrophage entry event is thought to involve transport of protein and not of DNA, and since the two events appear to compete for the same transfer apparatus, the *icm* gene products probably participate in a membrane structure that functions for both processes.

These findings broaden our concept of the mating bridge. More than simply a structural unit, it seems to be rather an interactive system that supplies energy for the transport of pili components and other proteins, export of transferring DNA/protein, and import of donor-specific phage DNA such as phage PRD1 DNA (53, 79). Conjugation and protein export must proceed without compromising the integrity of the bacterial cell wall. How this is done is only beginning to be understood for these processes. Interestingly, the PRD1 phage has a linear double stranded phage DNA genome with double 5' terminally attached proteins. Phage infection might then involve the incoming "transfer" of a protein-bound, double-stranded phage DNA through the conjugative mating bridge. This idea is consistent with experiments designed to determine the role of the IncP plasmid PRD1 infection. The need for the IncP plasmid can be obviated by electroporation of the phage genome, indicating that the plasmid components function only in the entry phase of infection (79).

3.1.4. DNA recircularization, complementary strand synthesis, and vegetative replication

Conjugative transfer of single-stranded DNA occurs with the 5' end "leading." This 5' end of the transferring strand is thought to begin its pilgrimage as part of the relaxosome at the membrane bridge, on the donor cell side, and end it at the membrane bridge on the recipient cell side. While it is associated with the mating bridge on the recipient side, recircularization presumably occurs, and the circular form is released into the recipient cell. The second nicking event at the mating bridge liberates one unit length of the plasmid and supplies the 3' terminal end for circularization. The TraI enzyme that catalyzes both nicking events also mediates this closing/ligation reaction. The process is best described in the RK2 system (30, 57).

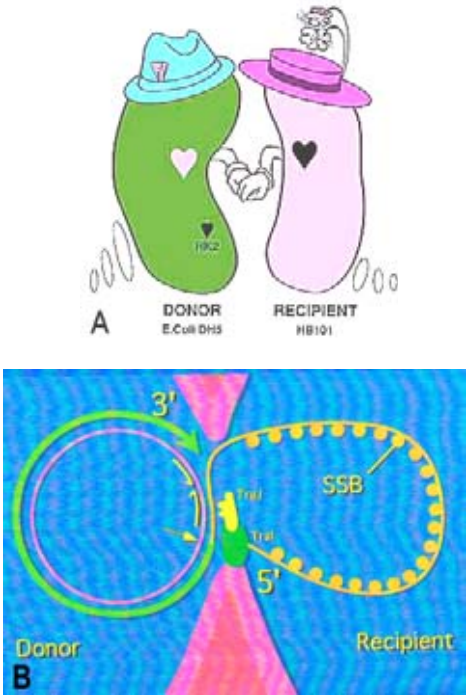

Figure 3. Donor and recipient *E. coli* bacterial cells and conjugative DNA transfer. 3A: Bacterial cell strains used experimentally in table 1 as conjugative donor recipient. The donor strain is *E. coli* DH5, carrying chromosomal DNA (larger heart) and conjugative plasmid RK2 (small heart). The recipient strain is *E. coli* HB101, with chromosomal DNA (large heart), but without plasmids. The RK2 plasmid will be transferred from the donor cell to the recipient cell by a replicative mechanism. 3B: Donor-to-recipient bacterial DNA transfer at the mating bridge, mediated by rolling circle replication which is initiated by 3' DNA extension at the nick site (shown by an arrow directed to the site joining orange- and green-colored DNA strand). The positions of the nicking enzyme complex TraI/TraJ and of the membrane proteins TraF/TraG are approximated and based on the model discussed in section 3.1. TraJ is thought to recognize the right arm of an imperfect inverted repeat sequence (two half-arrows) contiguous with the nick region, to favor TraI nickase activity and TraI covalent attachment to the freed 5' end of the DNA. Single-stranded binding protein (SSB) is bound to the newly-formed single stranded DNA which is the form of the DNA transferred into the recipient cell.

The model suggests that recircularization might occur before complementary straand synthesis is completed in the recipient cell, but in fact the timing of these two events is unknon, and they may occur simultaneously. In RK2 conjugation system transfers a 60 kb plasmid, so the likelihood of the 3' and 5'stranded ends coming into contact for ligation in the recipient cell by random interaction would be quite low. The conjugative ligation event thus provides efficiency by its unique form of nicking/closing activity. Following the first nicking event, the DNA 5' end is sequestered by covalent binding to the enzyme TraI, which is thought to act within the context of the mating bridge (figure 3). One unit length of the plasmid then enters the recipient, and is terminated by the second nicking event. The closing event is the ligation of the protein-bound 5' end to the newly obtained 3' end, the terminal ends kept in proximity by TraI. Substrate specificity distinguishes the first and second nicking events: the first nicking event has a double-stranded DNA substrate, and requires TraJ and TraI, while the second nicking event has a single-stranded DNA substrate and requires only TraI (57).

The described nicking/closing activities of TraI, to recircularize the transferred plasmid, has become the paradigm for other conjugative plasmids. Understanding this second nicking event of conjugative plasmids may be furthered by considering analogous systems with other molecular "goals." These systems include the Ti plasmid system, the rolling circle replication plasmid pT181, the rolling circle replication phage ϕX174, and the plasmid R1162, a mobilizable IncQ group plasmid closely related to RSF1010.

The Ti virulence plasmid system of *A. tumefaciens* infects plant tissue and induces tumor formation by conjugatively transferring a linear, single-stranded DNA segment into plant cells. In the Ti plasmid, there are two "*oriTs*" or "borders" in which nicking occurs (80). Within each of these *oriTs* is a putative nick region, but there are no inverted repeat sequences, as in RK2. Since the infecting DNA, which is the segment between the two *oriTs*, is transferred as a linear single strand, there is no recircularization event. Thus, the inverted sequences in RK2 may help coordinate the first nicking and second nicking/closing/recircularization reactions by favoring different activities of the TraI protein. The first nicking events occurs with a double-stranded DNA target, and the second on a single-stranded target. The target specificity could be facilitated by a change in DNA topology, as the inverted repeat more readily forms a hairpin structure in the single-stranded form. TraJ recognizes and binds the right arm of the inverted repeat of double-stranded DNA, when the first nicking event is favored, but not in the single-stranded hairpin form. Without the influence of TraJ interaction, TraI would be differently aligned with the nick region and recognize a different substrate. This second nicking event generates a second set of 5' and 3' ends. (The second 3' end would not participate in rolling circle replication as does the 3' end from the first nicking event.) The overall result is that TraI mediates the exchange the two 5' ends in relation to the 3' end of the second nicking event, which recircularizes the transferring DNA segment.

The two nicking events which initiate and terminate transfer of one plasmid molecule have been experimentally studied in the IncQ plasmid R1162, which has a nick region and *oriT* pattern which is very similar to the IncP pattern. A different termination nicking reaction for R1162 was engineered by placing a second *oriT* "downstream" from the first, in terms of the direction of DNA transfer. The termination/recircularization event at the second *oriT* was prevented if the inverted repeat within

this *oriT* was removed, consistent with the hypothesis described above (81).

How could TraI accomplish the second nicking/closing event if the active site remains covalently bound to the 5' end of the first nicking event? The answer is that there is good evidence for stereochemically distinct active sites for the distinct nicking events (82). One would predict that either another such active site, or another molecule, as in a dimer, would be needed to sequester the first 5' end for subsequent ligation.In the rolling circle replication plasmid pT181, the RepC protein is analogous to TraI of RK2. RepC acts as a dimer. With each complete replication cycle, the plasmid is nicked, replicated, and recircularized. The termination and recircularization irreversibly inactivates one subunit of the homodimer, converting it to a heterodimer form (RepC*) that can no longer initiate replication (83). It can, however, bind to the double-stranded replication origin, thereby acting as an inhibitor of initiation by blocking functional RepC binding. The goal of plasmid vegetative replication is to generate a controlled number of plasmid DNA molecules that can be maintained within a narrow copy number range. With pT181, the replication protein RepC becomes rate-limiting, so copy number is a function of the number of available active RepC proteins. This idea is consistent with the observation that the number of RepC dimers produced per cell approximates the number of plasmid molecules per cell (84).

In the bacteriophage ϕX174 replication system, the GpA protein is the analog of TraI of RK2 (31). GpA does not function as a dimer, but there are two active sites on the one protein monomer, each with an active tyrosine moiety for 5' binding. The GpA protein is then recycled, and thus does not become rate-limiting, as the tyrosine moieties alternatively repeat the cleavage and ligation events. Ultimately many DNA molecules are produced and contribute to a high phage titer. With a plasmid copy number of only 5 to 8 molecules per chromosome at 37^{o}C, RK2 conjugation probably transfers only a few of the full length 60 kb plasmid molecules. The IncP plasmid RK2, the ϕX174 phage, and the pT181 systems thus have distinctively different biochemical goals, suggesting that, for RK2, there should be a third mechanism. And in fact this is the case.

RK2 TraI neither acts as a dimer, nor does it have two active tyrosine moieties. The first nicking event, which constitutes relaxosome formation, results in the binding of the DNA 5' end to the TraI Tyr 22 moiety. TraJ first binds to the right arm of an inverted repeat adjacent to the nick region. TraJ then aligns TraI with the nick region in a way which favors Tyr 22 of TraI to act as the nucleophile. For the second nicking/closing event, there is another part of the TraI molecule, the putative second active site which is in fact highly conserved among analogous enzymes (57). This site has two histidines, separated by an isoleucine, and each appears to be involved in the termination nicking/closing event. Point mutations of these two histidines do not affect the initial event, that is, relaxosome formation. This is supportive of a role for these histidines in termination (53, 57). In termination, the TraI protein has three DNA "ends" with which to contend: the 5' end already attached to its Tyr 22, and the new 5' and 3' ends of the second nicking event. Since it is known that *in vitro* reactions using single-stranded substrates for nicking also result in the Tyr 22 binding to the nicked 5' end (57), the sites must facilitate a trade of the 5' ends. This promotes the ligation of the new 3' end to the first 5' as the latter is freed from the Tyr 22 moiety, resulting in recircularization. The newly generated 5' end can then presumably lead the transfer of a second plasmid length.

RK2 and certain other conjugative plasmids have their own plasmid-encoded primase molecules which are bound to the conjugating DNA and transferred from donor to recipient cell (70). The primase then primes lagging strand synthesis at multiple sites all along the DNA molecule, as does the *E.coli* primase DnaG prime lagging strand synthesis in RC replication plasmids. How many primase molecules are co-transferred with the DNA into the recipient cell is unknown. After the priming and the synthesis of short sequences, the host replication machinery is believed to complete the process. The *traC* primase gene of RK2 suppresses mutations in the *dnaG* primase gene of *E. coli*, and in wild-type *E. coli* recipients, *traC* is unessential. The recognition sequence for TraC of RK2 is most often d(TG) and sometimes d(CG) (29). The frequency of encountering these recognition sequences predicts that over a thousand priming events could start lagging strand replication of the 60kb RK2. Even if priming events were much fewer in number, the availability of transferred primase molecules could affect the speed and efficiency of RK2 conjugation. Alternate protein forms of the TraC primase, produced by alternate start sites within the coding sequence, are thought to impact host range by affecting transfer frequency to certain recipients. For example, in conjugation experiments using RK2 primase mutants in *E. coli* donors, reduced numbers of transconjugants were obtained with *Salmonella* and *Providencia* recipients (73).

Double-stranded, newly transferred RK2 plasmid DNA is maintained vegetatively by theta replication, beginning at the *oriV* and proceeding unidirectionally (70). Theta plasmid replication is a more complex form of replication than RC replication, requiring host cell and plasmid gene products. RK2 and other conjugative plasmids appear to have a conjugative host range that exceeds their replicative host range. A plasmid may therefore transfer into a bacterial recipient in which it cannot replicate, and subsequently integrate into the cell genome by recombination. The utility of bacterial strain engineering by means of conjugative nonreplicative "suicide" vector plasmids, carrying cloned genes that are selectable upon genome integration, documents the facility of this type of integration event. In pathogens carrying entire integrated plasmids, their existence may go unnoticed until the plasmid is conjugated into a host in which the plasmid can replicate. This scenario was recently demonstrated for a conjugative plasmid integrated into the *Haemophilus influenzae* genome by experimental transfer of the plasmid to *E. coli* (25). Thus, antibiotic resistance

genes first identified as chromosomally encoded may ultimately be deduced to be of plasmid origin.

3.2. Conjugative transposition

Conjugative transposons are nonreplicative genetic elements with self-transfer and mobilizing capabilities (85). Their transfer can occur within a cell, between chromosome and plasmid, and between cells, from donor to recipient. Conjugative transposons can be mobilized by other elements or plasmids and can mobilize a separate non-self-transmissible element or plasmid. As in conjugation, the transfer process of conjugative transposons requires cell-cell contact and is unaffected by the presence of DnaseI. Since conjugative transposons cannot independently replicate, they must be maintained in bacterial hosts by integration into genomic DNA or plasmid DNA. Like conjugative plasmids, conjugative transposons exist as double-stranded circular DNA molecules when independent of other DNA replicons. Unlike conjugative plasmids, conjugative transposons are not replicated in this circular state and are therefore segregated out during cell division, barring a timely integration event. Like conjugative plasmids, conjugative transposons encode and disseminate drug resistance among bacteria. Although conjugative transposons are considered broad-host-range, they are more often found among Gram-positive bacteria such as the streptococci and enterococci. Almost all conjugative transposons described to date carry genes for tetracycline resistance; some elements have been found to additionally carry resistance to kanamycin, chloramphenicol, or erythromycin (86).

Whereas conjugative plasmids were recognized before it was known that they were DNA molecules, conjugative transposons have been recognized relatively recently. In 1980, Franke and Clewell discovered in the chromosome of a *Streptococcus faecalis* strain a conjugative genetic element they called Tn*916* (87). Demonstrated as a mobile genetic element encoding drug resistance genes, Tn*916* and most other conjugative transposons have been given the traditional transposon Tn/*number* designation, without an added convention to distinguish the conjugative from the nonconjugative transposons. It has been suggested that the Tn designation for the conjugative transposons include a letter C (88) but the idea has not been quickly adopted. Some distinction would seem appropriate, because conjugative transposons are quite different from conventional transposons; they are not merely transposons with conjugative capacity. Five prominent observations align conjugative transposons in the conjugative plasmid category, more so than in the transposon category:

1. Transposons and conjugative transposons create distinctly different terminal ends in the target DNA upon insertion.
2. Transposons transfer only between DNA molecules within the bacterium while conjugative transposons transfer both intracellularly and intercellularly.
3. Transposons transfer from replicon to replicon entirely as double-stranded DNA, but conjugative transposons appear to transfer between cells as single-stranded DNA, after excision as a double-stranded circular intermediate.
4. Transposons require few genes to mediate transposition, while conjugative transposons have a large genetic commitment towards mediating the biochemical steps of cell-cell transfer.
5. Transposons and conjugative plasmids do not share homologies within genes encoding mobility functions, whereas conjugative transposons and plasmids do share genetic homology, organization, and functional analogy.

Of the above observations, the first three come from studies on the mechanism of conjugative transposition, and the last two observations come from a genetic examination. The discussion will focus on these two general categories, the mechanism of conjugative transposition and genetic findings, particularly in comparison to conjugative plasmids. The discussion will include another, similar mobile antibiotic resistance element, the non-replicating *Bacteroides* units (NBUs). By examination of these two non-replicative, "non-plasmid" systems, conjugative transposons and the related NBUs can be appreciated as well-equipped for drug resistance transfer. Such transfer may not be as efficient as the transfer of conjugative plasmids among Gram-negative bacteria, but nonetheless it supplies genes to bacteria that are not best served by conjugative plasmids. The teleological emphasis for these elements should be the virtues of effective conjugative transfer in a divergent niche of organisms. These elements uniquely contribute to the dissemination of antibiotic resistance genes among clinical bacteria, without the "luxury" of replicative maintenance. The context of conjugative transfer is what creates the parallel between these elements and conjugative plasmids, and is intended to distinguish this brief review of conjugative transposons from other, more general reviews (85, 86, 88).

3.2.1. The mechanism of conjugative transposition

To understand the mechanism of transposition of conjugative transposons, the mechanism of conventional transposons should first be considered. Insight into conventional transposition was furthered by an examination of the terminal ends and extrapolating to develop a model (89). These elements interact with the host target DNA to create a staggered, double-stranded cleavage. Upon insertion of the transposon between these ends, the overhanging sequences are filled-in, creating flanking sequence duplications which are exactly the length of the overhangs. These duplications are generally less than 12 base pairs. All classes and subclasses of conventional transposons share in this duplication of target sequences. These direct repeats of the flanking host target DNA are not to be confused with the transposon's own terminal inverted repeats which serve as enzyme recognition sequences mediating transposition.

Parenthetically, conventional transposition can be either replicative or nonreplicative in the sense that replication can occur as part of the excision event. The Tn*3* family of transposons and phage Mu are examples of

replicative transposons, while Tn*10* and Tn*5* are examples of nonreplicative transposons. The replicative transposons always conserve the DNA of the element in the donor DNA, at the time of excision, by concomitant replication. Neither replicative nor nonreplicative conventional transposons have circular intermediates.

Sequence analysis of the termini of different target areas of the prototypic conjugative transposon Tn*916* revealed that the mechanism of transposition for conjugative transposons was more complex, appearing even unpredictable, in comparison to conventional transposons. No duplication of target sequences was found. After analysis of the terminal sequences of a number of conjugative transposons, and comparing sequencing results obtained by electroporation (of circular intermediates), a somewhat complex model was developed (85, 89, 90). Unexpected findings included the discovery of terminal sequences containing mismatched base pairs, arising by virtue of the fact that the circular intermediate was not replicated after excision and was transient enough to avoid repair by mismatch repair systems (figure 4). These heteroduplexes, typically of 6 base pairs, could be identified by isolation of the circular intermediates in enough quantity to sequence.

Because of DNA homologies found in conjugative transposons and RK2, it has been inferred that the transfer mechanisms for conjugative plasmids and conjugative transposons are comparable. Transfer event similarities would apply after the conjugative transposon excises to become a circular intermediate. Adding weight to the argument is the observation that IncP plasmids can mobilize transfer of conjugative and mobilizable transposons, such as the element Tn*4399* (91, 92). With these observations in mind, the following mechanistic steps for intercellular conjugative transposition are offered.

1. excision/circularization of the conjugative transposon DNA by staggered double-stranded cleavage/closing, resulting in a 6 bp heteroduplex at the termini
2. cell-cell interaction and mating bridge formation
3. nicking at the *oriT* of one strand of the circular intermediate and relaxosome formation
4. transfer of the nicked strand through a mating bridge into the recipient cell
5. ligation/recircularization and complementary strand synthesis
6. targeting to a bent DNA segment within the recipient and staggered cleavage of target/transposon
7. integration, creating new junction heteroduplexes that are resolved by replication.

Since conjugative transfer by bacterial conjugation has been described at length in the previous section, and is presently thought to apply to conjugative transposition, the focus of this discussion will be on steps 1, 6 and 7: the excision/circularization, targeting, and integration events. Indeed, the biochemistry of cell-cell transfer in conjugative transposition has not yet been addressed experimentally, so the other steps cannot be reviewed at this point in time. What is known of conjugative transposition can, however, be presented as it impacts the prevailing conjugation model. The conjugative aspects of plasmids and conjugative transposons may then suggest that there is a similar conjugative event which can be packaged in different molecular backgrounds.

The excision/circularization event of conjugative transposons should not be confused with the ligation/recircularization of conjugation described above. Transposon excision is the process of clipping out the DNA of the transposon and then ligating together the flanking host DNA ends. Excision is mediated by transposon-encoded proteins Int and Xis, named after the analogous proteins of the bacteriophage λ system. There are similarities between the two systems, but one important difference is that the phage sequence has homology with its cognate bacterial insertion site. With conjugative transposons, the bacterial host target site is neither homologous to inserting element DNA nor is it a specific sequence, but there does appear to be an insertion preference for DNA regions that are intrinsically bent. In regard to the importance of the target site, the excision frequency appears to be related to the character of the bending at the integration site (93). In certain conjugative transposons, it has been found that the excision frequency can determine the number of circular intermediates; the number of circular intermediates can, in turn, determine the frequency of transfer (94, 95).

These observations suggest a way to distinguish conjugative plasmids and conjugative transposons with regard to a feature that can be measured in both systems: the frequency of transfer. The frequency of transfer in conjugative transposition must then be the product of the frequencies of all the constituent events. Such a multi-step process might be expected to result in lower overall frequencies of transfer than plasmid-mediated conjugation frequencies. The issue has not yet been addressed. Since conjugative transposition involves more biochemical steps than plasmid conjugation, a true comparison of only the conjugative frequencies might require the functional isolation of the conjugation event from among the other events in the process of conjugative transposition.

The Int and Xis proteins have been found to be encoded at the left arm of the related prototypic conjugative transposons Tn*916* and Tn*1545*. The Int protein is involved in both excision and integration. For excision, the Int protein binds to each arm region, including the adjacent flanking host DNA, and enzymatically cleaves at a sequence to create staggered ends with typically 6 bp overhangs (96). Ligation of the these non-base-paired ends generates the "excisant," which is the double-stranded, non-replicative circular intermediate generated by all conjugative transposons (figure 4). Conversely, integration cleaves the target region and the circular intermediate with 6 bp staggered ends, and ligates the target to the transposon ends. Prerequisite to these integrative steps in intercellular conjugative transfer would be the conversion of single-stranded to double-stranded DNA.

The Xis protein has been purified and used in nuclease protection assays and found to protect DNA sequences with a 10 to 11 bp periodicity over several helical turns (97). Thus it is believed to wrap the DNA

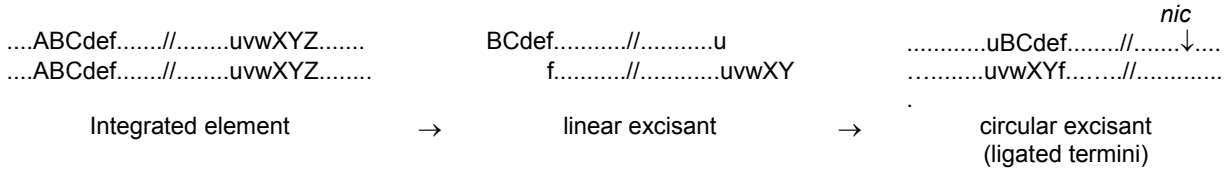

Figure 4. The generation of the heteroduplex DNA sequence upon excision and circularization of conjugative transposons. The flanking host sequences are symbolized by the upper case letters ABC and XYZ, and conjugative transponson sequences are symbolized by the lower case letters def and uvw. Staggered cleavage of the junction sequence creates two hypothetical overhangs, BCde and vwXY; subsequent ligation then creates the mismatching heteroduplex sequence of the circular intermediate.

around itself, histone-like, to facilitate DNA bending and to properly align Int. Xis thereby stimulates Int activity, and at the same time can favor excision over integration by inhibiting Int binding to a more distal site where it functions to mediate integration. In phage λ, the Xis protein is required for excision and binds only the right end, whereas the Tn*916* Xis protein has been shown by footprinting to bind specifically at both termini (96). For Tn*916,* Xis is not required for cleavage *in vitro* under certain conditions, such as in low salt (97). Int therefore appears to mediate both excision and integration, with Xis interacting to alter Int specificity, by favoring one of the two Int DNA binding sites and contributing to DNA melting (97).

Int has homology with the integrase family of recombinases. In Tn*916*, Int mediates cleavage with a polarity that is also seen in phage, generating a free protruding 5'OH group and a transient covalent linkage to the 3' phosphate DNA end. The Tn*916* terminal ends contain imperfect inverted repeats, within an AT-rich environment, which presumably facilitate the formation of a postulated looping structure (85). Such looping would allow both ends to be bound in close proximity, for concomitant double cleavage and circularization. Int is thought to recognize perfect direct repeats within each of the termini of the conjuagtive transposon, employing DNA binding domains that cleave within the flanking host coupling sequences as it catalyzes cleavage and joining (98).

The Int of conjugative transposons is unique among recombinases in mediating cleavage of DNA substrates containing its transposon termini and a segment of the adjacent bacterial host sequences. The resulting excisant is created containing short sequences from host DNA sequences of the donor bacterium (figure 4). These sequences have terminal overhangs that are not homologous but which come together to form short heteroduplex sequences upon circularization. After integration into the recipient bacterium, a coupling sequence is then found at either junction of the transposon/target DNA. The significant observation here is that the coupling sequences are not found at both junctions of the newly integrated element. These findings lead to the following questions. How does just one of the two coupling sequences enter the recipient cell? If only one host coupling sequence is transferred, is it randomly chosen from either end?

We can attempt to answer these questions from what we know of plasmid conjugation. We would predict that the "choice" of which flanking host sequence is transferred is according to which DNA strand is nicked, that is, which strand of the excisant contains the nick site. If the transfer occurs as a single strand, that single strand would encode only the "top" or "bottom" strands of the donor coupling sequences, but not both. The excisant ligation creates the heteroduplex from the staggered termini of each flanking coupling sequence, the top from the left and the bottom from the right, for example. The other strand of the donor excisant is not nicked or transferred, so only one flanking terminus of the excisant's heteroduplex is transferred. No heteroduplexes are transferred if the transfer is of a single-stranded DNA molecule. The excisant is the molecular form which has been analyzed and found to have heteroduplex ends, so this model is consistent with relevant findings. The hypothetical sequence scenario shown in figure 4 illustrates these events. The upper case letters indicate coupling sequences from the host DNA, ABC or XYZ, the lower case letters indicate the DNA sequence of a conjugative transposon termini, def or uvw. In the example, the hypothetical 4 base pair mismatching sequence created by the staggered cleavage forms the heteroduplex BCde/*vwXY* upon excision and ligation (figure 4).

The nick site (*nic*) is on the top strand in the example, so the top sequence would be transferred, leaving behind the sequences of the bottom strand including those forming the heteroduplex, in this case the right-hand host coupling sequence XY. Thus the answer to the first question (how it is that one but not both host flanking sequences is transferred) is answered by the described consequences of the single-stranded DNA transfer model. The second question (how the one flanking sequence is chosen over the other) is answered by the observation that insertion is not orientation specific. This means that the conjugative transposon could insert "upside down," with the nick site on the bottom strand and the other flanking host coupling sequence linked to the nicked and transferred strand. A lack of observed "bias" in insertion orientation is consistent with the model (85).

The excision event of conjugative transposons probably require some host factor(s), in light of the fact that the Gram-positive organism, *Lactococcus lactis*, can serve very well as a recipient but cannot serve as a donor for Tn*916* transfer (85). Once the element integrates in this organism, apparently it cannot excise. This organism, not a pathogen, is therefore a dead end for conjugative transposition. Studies of the biochemical requirements for excision of the Tn*916* circular intermediate form have not yet revealed host factors required *in vivo*, so the function missing in *L. lactis* is unknown.

Targeting to the integration site has been studied in some detail. Targeting sequence preference is neither by sequence specificity nor according to areas of AT-richness, but apparently to areas of DNA bending, at least in the Tn*916* family. In one study of all target sites obtained in one conjugative transposition experiment, the most commonly found target had the following sequence (89):

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TTTTATAAT..........AAAAAGAAAT
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The dotted line indicates where the conjugative transposon had inserted. Before integration, this target sequence has As and Ts spaced over two helical turns that would produce a bent DNA molecule with the As on the outward side of the curvature. This region would also be underwound in the central area, conceivably to facilitate Int interaction. Int would probably be at low concentrations in the recipient directly after transfer, since the window of time for its production and availability would be limited to the time after transfer and complementary strand synthesis and before integration.

Integration *in vitro* by Tn*916* Int cleavage has shown that Int resembles other integrases, in particular those integrases which form transient covalent linkage between the protein C-terminus and 3'-phosphate of the cleaved DNA (98). The N-terminal region of Int binds to directly repeated sequences at the conjugative transposon's termini, while the C-terminal region binds to the regions containing inverted repeats further out on the termini, and is thought to interact with the targeted host sequences (96). While binding patterns appear to be like those of bacteriophage λ, the resulting nucleoprotein structures are different, the phage structure the more complex (85). For Tn*916,* the cleavage, strand exchange, and closing reactions are all mediated by Int (98).

The substrate for integration functions has been deduced by comparing integration junctions with those obtained by transformation of *Bacillus* protoplasts with isolated double stranded circular intermediates (90). With circular intermediate DNA transformation, followed by integration, the resulting structure has coupling sequences at the junction sites from the donor cell at both junctions of the transposon/target. This contrasts with results with conjugative transfer followed by integration, in which case the coupling sequence is found at only one junction.

These results, that different integrated molecules are generated by transformation and conjugation, is consistent with the rolling circle, single-stranded DNA transfer model for conjugative transposition (see above). The necessity of complementary strand synthesis of the transferred single-stranded DNA in the recipient cell can explain how only one strand of the heteroduplexes is preserved at the integration junction. The other possible explanation, that a double-stranded molecule is conjugatively transferred, invokes a strand-specific mismatch repair model, to be consistent with the observed preservation of only one junction host coupling sequence (85). A strand-specific mismatch repair seems very unlikely, especially in light of the other observations. Conjugative transposon heteroduplex analysis has thus provided good supporting evidence for a plasmid conjugation model involving single-stranded DNA transfer, and, by inference, for rolling circle replication in the donor to generate the single strand that is transferred.

Conjugative plasmids can mobilize conjugative transposons, and the converse, that conjugative transposons can mobilize mobilizable plasmids, is also true. These observations are consistent with the notion that the two types of conjugation systems are not only similar in mechanism, but have functional complementarity. Cross-complementation of mutant derivatives in the two systems would experimentally further the parallels. If the transfer mechanisms of conjugative transposons and of plasmids are ultimately shown to be functionally the same in the essential steps, these connections between the two systems would support a unified model for both plasmid-mediated and transposon-mediated conjugative transfer.

The tentative steps for conjugative transposition #2 through #5 (above) are based on conjugative transfer events of bacterial conjugation: cell-cell interaction and mating bridge formation, DNA strand-nicking of the plasmid or circular intermediate, strand transfer to the recipient, ligation and recircularization, and complementary strand synthesis. After excision, the double-stranded non-replicative intermediate apparently undergoes a nicking event within an *oriT* region that is similar to that of conjugative plasmids. These *oriT* regions have been identified by sequence similarities to known *oriT* regions, and appear to be located at sites which are distant from the terminal ends involved in integration and excision (91, 93, 99). It is noteworthy that the broad-host-range potential of many of these elements suggests that few host products are required for integration. Most conjugative transposons appear to be broad-host-range, but there are some that appear to have a more limited host range (85). Little is known about the factors determining host range in any conjugative transfer system, plasmid or conjugative transposon, but it is expected that different systems have host range differences which affect frequency, if not feasibility. What is germane to this discussion of antibiotic resistance is that the absence of the replication function in conjugative transposons impacts host range. Without replication, the host range limitations of plasmids in recipient bacteria which cannot support the plasmid's replication is obviously not an issue. Therefore, the element's replication-deficiency status could in fact be viewed as factor which indirectly extends the host range. For example, *E. coli* and *Bacteroides* could

potentially exchange such elements, although plasmids native to each bacterium cannot replicate in the other organism (43).

3.2.2. Non-replicating *Bacteroides* units

Non-replicating *Bacteroides* units, called NBUs, are integrated DNA elements that can excise and transfer only if mobilized by *trans*-acting conjugative transposons carried in the same cell. With less functional capability, NBUs are smaller, only 10 to 12 kb, but they often encode tetracycline resistance determinants and have been found to carry cefoxitin resistance (100). A minimum mobilizable *oriT* region (called *mob* in NBUs) of about 3 kb has been established (101).The mobilization of NBUs by related conjugative transposons can be seen as analogous to the mobilization of non-self-transmissible plasmids, such as the IncQ plasmids by IncP plasmids. The NBU *oriT/mob* region has been experimentally mobilized by IncP plasmids (99). Although NBUs have so far been found only in *Bacteroides*, they have been experimentally mobilized to *E. coli* (86). The relevance of NBUs to antibiotic resistance transfer is that, although they cannot self-transfer, they can be mobilized by conjugative transposons and by conjugative plasmids. The existence of these elements therefore supports the view that there is a virtual molecular continuum of genetic elements that transfer antibiotic resistance. The comparative approach can then be of great value in understanding molecular specialization, host range, and other transfer phenomena of these elements.

Mobilization of one particular NBU, called NBU1, by IncP plasmids might be facilitated because of nearly identical nick regions in the systems that have been employed. This would mean that not only the mobilizable functions of the IncP plasmids would be involved in transfer, but that possibly the cognate nicking enzyme apparatus, encoded by *traI, traJ,* and *traK* (see above), could also be instrumental. This possibility is offered here as the fruit of examination of the published *oriT* region of NBU1 (101, 102). The probable nick region of the NBU1 element lies 220 bp upstream from its *mob* gene, and on the other strand relative to the *mob* coding strand. It is not likely to be the sequence that lies 360 bp upstream from *mob,* the sequence noted by the investigators (102). Mutation analysis was not carried out to identify any nick region, so the suggested sequence was proposed by putative sequence homology to some unidentified nick region. The sequence that lies 220 bp from the *mob* gene would be the nick region because it reveals near identity with the nick regions of rolling circle replication plasmid pTC-FC2 and of RK2. The NBU1 sequence fits well into the IncP family (table 5), in light of the fact that nick region families have been derived by sequence homology (31). These three nick regions are shown below, the arrows indicating nick sites. The variant nucleotides to the left of the IncP nick site are noted with dotted lines:

pTF-FC2	TCATCCTG$^{\downarrow}$TA
	:
NBU1	TCAACCTG$^{\downarrow}$TA
	: : :
RK2	CTATCCTG$^{\downarrow}$CC

The genes encoding the Mob nicking proteins of NBU1 and pTF-FC2 might conceivably complement each other, since there are regions of amino acid sequence similarity and a similar spacing pattern between the consensus regions (101, 102). The mobilization of NBU1 by RK2 can be viewed as the mobilization of the NBU relaxosome, analogous to RK2 mobilization of plasmids such as RSF1010. While conjugative transposons and their mobilizable NBU-type elements are non-replicative conjugation systems, they are conjugation systems analogous to those of conjugative and mobilizable plasmids. The following genetic addition equations provide a way to summarize these statements. The intent is to illustrate the functional concepts, not the historical process.

1. mobilizable, non-self-transferable plasmid + conjugative transfer genes = conjugative plasmid
2. NBU + conjugative transfer genes = conjugative transposon
3. conjugative transposon + replicon = conjugative plasmid

4. DISSEMINATION OF RESISTANCE TO BETA-LACTAMS AND AMINOGLYCOSIDES

From the earliest studies of genetics in bacteria, it was recognized that the laboratory demonstrations of transduction, transformation, and conjugation represented a great potential for genetic flexibility in natural environments. The question asked then continues to be addressed today: what does all this genetic potential actually accomplish in natural settings and under commonly encountered selective pressures? In answering this question, the study of antibiotic resistance gene acquisition has been, and will probably continue to be, the best model system for what happens in nature under heavy selective pressure. Further, the study of resistance gene acquisition continues to provide the means to discover variations on the theme of genetic flexibility brought about by mutation, transformation, and conjugation. Integrons, NBUs, and other carriers of antibiotic resistance genes demonstrate the creative expression of bacterial genetic flexibility and adaptation.

The present discussion of conjugative transfer is certainly relevant to the dissemination of the genes for resistance to the β-lactams and aminoglycosides. Most of the dissemination of resistance to these antibiotics has in fact been mediated by conjugative transfer. The prototypic plasmids for the study of bacterial conjugation have been the IncP and IncF plasmids. Although the IncF plasmids, in particular the F plasmid, provided the model system of choice for years in studying the particulars of conjugation, the details of DNA preparation for conjugation have been in recent years worked out in greater detail in IncP plasmids. It may be safe to say at this point that the IncP plasmid RK2, also called RP4, has become the bacterial plasmid conjugation prototype. Much of the credit for these recent advances must go to Erich Lanka and his many collaborators.

RP1, RP4, R68, and RK2 were part of the original group of large IncP plasmids called the

Birmingham group. Molecular analyses were not feasible when they were first discovered, so it was not until later that these plasmids were declared molecularly indistinguishable. The drug resistance gene-carrying plasmids of the 1960s and 1970s were commonly named according to the host pathogenic bacterium, or in some cases, the host patient from which the bacterium was isolated, with an initial "R" to signify drug resistance-encoding. The Birmingham group term referred to a burn unit in the Birmingham Accident Hospital, Birmingham, England, where nosocomial organisms that carried this plasmid were isolated in the late 1960s and early 1970s (103).The plasmid, most often called RK2 or RP4, carries the resistance genes for three antibiotics that were then the commonly used antimicrobials of the day: the aminoglycoside kanamycin, penicillin, and tetracycline (103). These resistance genes had apparently been transferred by this large conjugative plasmid among patient isolates of *Klebsiella* (as RK2) and of *Pseudomonas* (as RP4). Since the transfer of this plasmid was demonstrated between *E. coli* and these organisms, the term "broad host range" was used to distinguish RK2 from the narrow host range transfer of IncF plasmids (table 3). The fact that the TEM β-lactamase gene encoded by RK2 is expressed well in the enterics, as well as in the non-enterics *Pseudomonas, Neisseria,* and *Haemophilus,* is circumstantial evidence for the conjugative transfer of this gene among these strains in nature.

Two different clinical examples of the current and ongoing importance of conjugative plasmid transfer of resistance to antibiotics including β-lactams and aminoglycosides are the pathogens *K. pneumoniae* EK105 and *Yersinia pestis* 17/95 (3, 104). Both of these organisms are multiresistant, virulent bacteria causing infections of high mortality. The *Klebsiella pneumoniae* EK105 strain carries a mobilizable plasmid and a large conjugative plasmid encoding between them resistance to amikacin, ampicillin, chloramphenicol, kanamycin, streptomycin, tobramycin, netilmicin, oxacillin, gentamicin, and mezlocillin (3). The resistance profile of this organism, causative agent of neonatal meningitis and septicemia, leaves the infection nearly untreatable and often fatal.

A similar situation has been found with a strain of the causative agent of plague, *Yersinia pestis*. After an era of silence in regard to epidemic and endemic infection, there has been an increase in the numbers of cases of plague reported in India, Africa, and North America. The organism has been regarded as uniformly susceptible to antibiotics active against most Gram-negative bacteria. The emergence of strain *Y. pestis* 17/95 demonstrates that this can no longer be presumed, since it encodes resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, spectinomycin, sulfonamides, tetracycline, and minocycline (104). These antibiotics include those recommended for prophylaxis and therapy. Experimental conjugative transfer of the resistance determinants from *Y. pestis* 17/95 and from a similar strain to *E. coli* was shown to be mediated by a large plasmid (104).

The broad host range transfer system of IncP plasmids has been employed to engineer conjugation systems that transfer shuttle plasmids between Gram-negative and Gram-positive bacteria (12, 38, 105). Plasmids from Gram-negative organisms generally cannot replicate in Gram-positive organisms, while some plasmids of Gram-positive organisms do replicate in Gram-negative organisms (106). Therefore, in most conjugation experiments from Gram-negative to Gram-positive bacteria, Gram-positive-specific replication regions must be added to the shuttle vectors to ensure plasmid replication in the recipient. It is not yet established if this kind of transfer, from Gram-negative to Gram-positive organisms, occurs in nature, but there is evidence for the reverse event, which might be the more expected (107). There are many conjugative plasmids native to Gram-positive bacteria, and the lineage of antimicrobial drug resistance genes has been postulated to involve gene transfer from the soil organisms, first transferred to the Gram-positive bacteria, and then from Gram-positive bacteria to the Gram-negative bacteria (108). In 1965, in separate reports, β-lactamase genes were first found to be plasmid-encoded, from strains of *S. aureus* and of *E. coli*, and, in 1970, a 53 kb conjugative plasmid encoding resistance to both aminoglycoside and β-lactam antibiotics was isolated from *Staphylococcus aureus* (109, 110). Thus, the natural history of the emergence of bacterial resistance to the aminoglycosides and β-lactamases by means of conjugative transfer may have followed the expected paradigm:

Soil microorganisms → Gram-positive bacteria → Gram-negative bacteria

The history of transferable drug resistance parallels the history of β-lactamase resistance, and the history can be traced through bacterial genera, but the missing information is the identity of the plasmid transfer systems actually mediating the resistance dissemination. Using RK2-based vectors to transfer markers from Gram-negative *E. coli* bacteria to other species, recipients have included all of the Enterobacteriaceae, *Acinetobacter, Bacteroides*, *Aeromonas, Alcaligenes, Agrobacteria, Bordetella, Caulobacteria, Legionella, Mycobacteria, Neisseria, Pseudomonas, Vibrio,* and many other bacterial species (37). These studies illustrate the potential of conjugative transfer of antibiotic resistance genes in nature by one type of plasmid, but the present-day clinical relevance of this conjugative potential is unknown.

In recent years, there has been less interest in correlating the conjugative plasmid Inc group with a particular antibiotic resistance gene. Surveys were formerly done to correlate antibiotic resistance genes with plasmid carriers, by the Inc group of the plasmid. For example, the genes encoding resistance to kanamycin were found on conjugative plasmids of the IncI, IncT, IncD, IncH, IncX, IncP plasmid groups (111). What could be the usefulness, in terms of the clinical resistance problem, of correlating Inc group with a given gene? The incompatibility (Inc) groups are based on replication similarities, such that plasmids of the same Inc group cannot be maintained together in the same bacterial cell. This is due to similarities in replication and maintenance entities that are not distinguished at the time of cell division, with the result

that one type (Inc group) of plasmid is diluted out of the bacterial population. A particular conjugation system may not always be correlated with a particular Inc group. Although such a correlation has often been observed, and has been the basis of surveys, plasmids are viewed more often now as a mosaic of genetic subsets that are interchangeable to some extent. A case in point is the Ti plasmid, which carries two distinct and specialized conjugation systems on the same plasmid (40). In RK2, there is an overlap of regulation of the conjugation system genes and replication, but it is unknown if this type of co-regulation occurs in other plasmids (30). There has not yet been an exhaustive survey of the conjugative transfer genetic groups with reference to Inc grouping and other plasmid characteristics. The question that could be addressed with this type of information is whether there are patterns of plasmid transfer system grouping with the Inc group, and with particular antibiotic resistance alleles. The value of studying conjugative transfer in reference to resistance genes is to foresee potential strategies for new targets for clinical treatment. Certain plasmid systems may have a commonly held property that could be targeted. Observations that initially appear to be subtleties may provide a key to thwarting plasmid-mediated spread of resistance genes.

How the normal flora might transfer drug resistance genes to pathogens in *vivo* has been addressed, using *S. epidermidis* as the donor and *S.aureus* as the recipient. Two different plasmids encoding resistance to the aminoglycoside gentamicin were transferred on human and mouse skin, and transfer on skin was at a higher frequency than *in vitro*, on laboratory media (112). Thus, natural host habitats may well promote bacterial conjugative transfer, as has been inferred over the years, especially in hospital environments where selective pressures are most prevalent.

"By long forbearance a ruler is persuaded."

Hebrew proverb

5. SUMMARY AND CONCLUSIONS: BREAKING THE CYCLE OF RESISTANCE

Recognition of the prevalence of antibiotic resistance genes has followed the magnitude of the problem, in recent years if not from the beginning. Early in the history of antibiotic resistance, the clinician and public at large cherished the thought that infectious disease had been conquered by penicillin and other antibiotics. There was indeed a time when sexually transmitted diseases would be inadvertently treated with a single dose of penicillin, intended for strep throat. Some measure of complacency was not unreasonable, for a season. Almost from the beginning, however, there were "alarmists" who had foresight enough to anticipate what is now undeniable, that the antibiotic resistance problem is rapidly becoming a worst case scenario. The alarm has predictably resulted in an increase in the human investment side of the cycle of resistance, towards an increased rate of development of new antibiotics (figure 1). These efforts are of course necessary, but other, more effective and long-term solutions are needed to address the problem and break the cycle of resistance. A deliberate decrease in clinical dependence on antibiotics is a consideration that has some merit, but it can only be of short-term and limited value.

This survey of the common themes in conjugative transfer as mediated either by plasmids or conjugative transposons has presented some of the information gleaned from a very limited number of prototypic systems studied in the most detail. While the plasmid systems have been available in some form for study for more than 30 years, the conjugative transposons are relatively new objects of study. Studies of conjugative transposons have focused on mechanism of transposition and not on conjugative transfer. What little is known, however, suggests that the two systems, found in different bacterial populations in nature, have similar cell-to-cell conjugation properties. This potential for ideological unification has therefore been offered here, because the strength of the comparative approach can be to the advantage of future investigators. Comparative studies can inform the basic science of conjugation and be used to address the problem of antibiotic resistance gene dissemination by conjugative transfer. One conclusion that might be surmised from this discussion is that the application phase, that is, the actual prevention of drug resistance dissemination, is not imminent.

Detailed tracking of genes disseminated by conjugative transfer is needed, to sort out what genes have been disseminated by which conjugative plasmids. The different alleles encoding resistance to tetracycline have been catalogued and correlated to bacterial species and to genetic element, in order to infer genetic transmission routes (113). Tracing the "lineage" of such genes can become very complex. Thorough study of tetracycline resistance determinants has involved analysis of genes distributed among 32 Gram-negative and 22 Gram-positive bacteria (113). The complexity itself demonstrates the severity of the problem. This type of categorization reveals the wealth of resistance genotypes that can evolve in response to one antimicrobial agent of widespread use, in this case, tetracycline.

Genetic analysis has not been done as extensively for any other antibiotic, giving this tetracycline work distinction as a model study. With similar investigations of other antimicrobials, molecular family trees could be drawn to illustrate the patterns of spread of resistance genes. Conjugative plasmids and other conjugative genetic elements must be surveyed among current multiply drug resistant clinical strains, and the basic science of the more prevalent systems pursued, to point to possible anti-transfer strategies. The basic science of plasmid conjugation has proceeded with tangible increases in understanding, but the accumulated data still depicts a rather rudimentary model. Plasmid conjugation is typically regarded as a uniform system common all such plasmids. Important differences have not yet been attributed to teleology. For example, the nature and virtue of the difference between broad host range and narrow host range are unknown, even after these 30 years of investigation.

An entirely different approach to the antibiotic resistance problem is the development of gene therapy directed toward infectious disease. Most of the studies to date

have been antiviral strategies, but bacterial diseases are expected to be targeted. Gene therapy could involve gene delivery by the conjugative plasmids of bacteria, as well as by the other more established gene delivery systems such as those using viral vectors, bacterial invasion, and transfection methods. With the new concept of gene delivery by conjugative transfer, the transfer from engineered bacteria carrying the therapeutic transferable genes would be directed to the host mammalian cells as recipients (41). This type of directed transfer could include immunotherapy and vaccine development, adding a new dimension to traditional approaches.

Traditional vaccines for *Haemophilus influenzae* and more recently for *Streptococcus pneumoniae* serve to reduce antibiotic resistance selective pressure for those organisms, more than any other approach (114). This is especially true because they have been directed against common pediatric infectious disease organisms of increasingly problematic antimicrobial resistance. Vaccine development can therefore become a way out of the cycle of resistance for certain organisms, and new technologies such as engineered gene transfer could broaden the effectiveness of vaccine strategies.

Vaccine development will not be a feasible approach, however, for two groups of organisms: the emerging new bacterial pathogens, and the organisms considered part of the normal, even beneficial, microflora. For these groups of organisms, antibiotic treatment will continue to be critical, and research for new agents necessarily ongoing. Conjugative transfer approaches might be appropriately targeted to these bacterial recipients, especially those in this second group, the normal flora, which includes enteric organisms such as *E. coli, Klebsiella,* and *Enterobacter*. Conjugation to the mammalian host cells as a form of gene therapy could also be attempted, and be customized for therapies requiring long term gene maintenance and expression. This would be a multipurpose approach for combating both the infection at hand and the subsequent spread of antibiotic resistance genes. Further characterization of clinically relevant conjugative systems, and of the potential bacterial donors, is also needed to develop these strategies. Combined with early diagnosis and treatment, such creative and increasingly complex additions to the antibacterial armament should allow us to adequately address the problem of the cycle of resistance.

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