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MINIREVIEW

Why Are Antibiotic Resistance Genes So Resistant to Elimination?

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EASY TO GET AND HARD TO LOSE: THE SPREAD AND PERSISTENCE OF ANTIBIOTIC RESISTANCE GENES

The euphoria produced by the discovery of antibiotics led to confident predictions that bacterial diseases would soon be conquered and could thus be safely forgotten, leaving scientists free to attack other pressing health problems such as viral diseases and cancer. Instead of witnessing the disappearance of bacterial diseases, however, we are now experiencing a resurgence of them, both in hospital and in community settings. To make matters worse, bacterial pathogens have become increasingly resistant to a variety of antibiotics. A major driving force behind the increase in resistant strains is the ease with which bacteria can acquire resistance genes, even from distantly related genera. Much of what has been written about gene transfer elements has focused on elements acting in isolation. Yet, gene transfer elements can—and often do—hunt as a pack, by interacting with each other in a variety of ways that enhance their collective ability to transfer resistance genes. This interactive capacity needs to be taken into account when considering the potential for horizontal transfer of resistance genes. One aim of this minireview is to consider the consequences of cooperative behavior between different gene transfer elements. The characteristics of the different elements involved in the spread of resistance genes are summarized in Table 1.

Although the acquisition of new resistance genes is an important factor in the increasing incidence of resistant strains, it is only part of the resistance story. A critical, but often underappreciated, feature of resistance gene transfer elements is their stability. Their ability to adapt rapidly to new hosts so that they are not readily lost even in the absence of antibiotic selection may explain why increases in resistance can be so hard to reverse.

The hope that the cessation of the use of a particular antibiotic will cause resistance to that antibiotic to disappear is proving to be illusory. It is true that decreased use of an antibiotic is usually accompanied by some decrease in the incidence of resistant strains. For example, in a country-wide Cuban program to control the use of certain classes of antibiotics, impressive decreases in resistance to most of the restricted-use antibiotics occurred (7). Similarly, a decrease in penicillin-resistant strains of *Streptococcus pneumoniae* was seen in Hungary after doctors drastically reduced their use of penicillin for the treatment of pneumococcal infections (17).

As encouraging as such reports may seem at first glance, closer inspection reveals a different and more ominous message. Although the incidence of resistant strains may drop, it seldom falls to zero (7, 17). This leaves a residuum of persistently resistant strains that can rebound rapidly to become the predominant strains if antibiotic use is resumed. An example of this phenomenon has been provided by Gerding et al. (11), who described the rapid return of resistance to gentamicin and tobramycin when gentamicin was reintroduced after amikacin use was discontinued in a hospital. Unfortunately, relatively few studies of this type have been done, so it is not clear whether rapid rebound of resistance will occur with all types of antibiotics or only with some antibiotics.

Another troubling message comes from studies of nonclinical isolates. Calva et al. (8) found high levels of resistant strains of enteric bacteria in the feces of children from urban areas of Mexico. In this case, episodic use of antibiotics, which in Mexico are available without prescription, may be sufficient to maintain high levels of resistant strains. Another study that compared antibiotic resistance patterns in bacteria from feces from rural and urban Mexican children (2) showed that in general the bacteria from rural children were less resistant than those from urban children, except in the case of antibiotics used agriculturally. There is still relatively little information about the impact of agricultural use of antibiotics on resistance levels. Finally, a study of groundwater isolates from rural Tennessee revealed unexpectedly high levels of resistant enteric bacteria (16). It is difficult to rule out some sort of antibiotic contamination of the groundwater. Nonetheless, it is noteworthy that although chloramphenicol has rarely been used in the United States over the past decade, nearly 17% of the coliforms in the groundwater have been found to be resistant to chloramphenicol. Any successful attempt to curb the spread of resistance will have to take into account the fact that resistance genes and the transmissible elements that carry them are hard to lose as well as easy to get. What we do not know and desperately need to learn is whether the persistence of resistant bacteria in the environment is due to low-level antibiotic contamination, some nonantibiotic selection, or simply the stability of the resistance genes and transfer elements.

This minireview surveys some recent work on the "easy-to-get" and "hard-to-lose" sides of the resistance gene transfer equation. The purpose of this minireview is not to provide an exhaustive coverage of these topics, a task which would require much more space than is available in a minireview. Rather, we want to provoke a reassessment of some widely held views about antibiotic resistance. Accordingly, only selected examples and selected references are provided to illustrate the points being made. Where possible review articles rather than original papers are cited. Another aim of this minireview is to highlight the fact that virtually all of the studies of resistance patterns done to date have focused on one narrow group of

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TABLE 1. Characteristics of different elements involved in resistance gene spread

Element	Characteristics	Role in spread of resistance genes
Self-transmissible plasmid	Circular, autonomously replicating element; carries genes needed for conjugal DNA transfer	Transfer of resistance genes; mobilization of other elements that carry resistance genes
Conjugative transposon	Integrated elements that can excise to form a nonreplicating circular transfer intermediate; carries genes needed for conjugal DNA transfer	Same as self-transmissible plasmid
Mobilizable plasmid	Circular, autonomously replicating element; carries gene that allows it to use conjugal apparatus provided by a self-transmissible plasmid	Transfer of resistance genes
NBU[a]	Integrated elements that cannot excise and transfer themselves; can be triggered to excise and transfer by conjugative transposons; transfer intermediate is a nonreplicating circle carrying a gene that allows the NBU to take advantage of the conjugal transfer apparatus of a conjugative transposon	Transfer of resistance genes
Transposon	Can move from one DNA segment to another within the same cell	Can carry resistance genes from chromosome to plasmid or vice versa
Gene cassette	Circular, nonreplicating DNA segments containing only open reading frames; integrates into integrons	Carry resistance genes
Integron	Integrated DNA segment that contains an integrase, a promoter, and an integration site for gene cassettes	Forms clusters of resistance genes, all under the control of the integron promoter

[a] NBU, nonreplicating *Bacteroides* unit.

bacteria: *Escherichia coli* and its close relatives. *E. coli* and other enteric organisms are significant causes of human disease, but many important pathogens are members of other phylogenetic groups of bacteria. Information derived from studies of enteric bacteria may not be representative of all pathogenic bacteria. In fact, as resistances are being studied in bacteria outside the *E. coli-Pseudomonas* phylogenetic group, it is becoming clear that what holds for the enteric bacteria does not necessarily hold for members of other phylogenetic groups. To make this point, we have placed considerable emphasis on the *Bacteroides* spp. and on the gram-positive bacteria, examples of bacteria from phylogenetic groups other than the one in which the enteric bacteria are located. Also, *Bacteroides* spp. and gram-positive bacteria are the numerically predominant bacteria in the human colon and may act as very important reservoirs for resistance genes.

BROAD-HOST-RANGE RESISTANCE GENE TRANSFERS OCCUR READILY IN NATURE

Early reports of very broad host range gene transfer events, e.g., between gram-negative and gram-positive bacteria (9), were viewed by many people as laboratory curiosities that could not possibly occur in nature. Yet, there is a growing body of evidence that the horizontal transfer of resistance genes between bacteria of different species and genera occurs easily and frequently in nature, even between bacteria that normally reside in different sites. Some of the evidence comes from animal studies documenting gene transfer in the intestinal tracts of rodents (10, 15), but the most convincing evidence comes from finding virtually identical copies of the same resistance gene in distantly related bacteria isolated from the intestinal tract or the external environment (Fig. 1). For example, alleles of *ermF* have been found both in oral spirochetes and in human colonic *Bacteroides* spp., and alleles of *ermG* have been found both in colonic *Bacteroides* spp. and in a *Bacillus* sp. from soil (20). Alleles of *tetM* have been found in a variety of gram-positive and gram-negative bacteria (18–20). The percent identity of the genes found in different bacterial hosts is too high (usually over 95%) to be the result of convergent evolution and must have occurred via horizontal gene transfer. Moreover, many of the resistance genes found in distantly related hosts are being expressed in these hosts. Thus, resistance genes themselves, like the elements that carry them, can have a very broad host range. These studies have forced us to revise our definition of what "broad host range" means. At one time, transfer between *E. coli* and *Pseudomonas* spp. was considered to be broad-host-range transfer. Today, these would be considered narrow-host-range transfers, compared to transphylum transfers, such as those between gram-positive bacteria and enteric bacteria or between members of the *Bacteroides* phylogenetic group and the enteric bacteria.

Presumably, broad-host-range transfers such as those illustrated in Fig. 1 are being mediated by conjugation. In cases in which the genes are on known conjugal elements, as was the

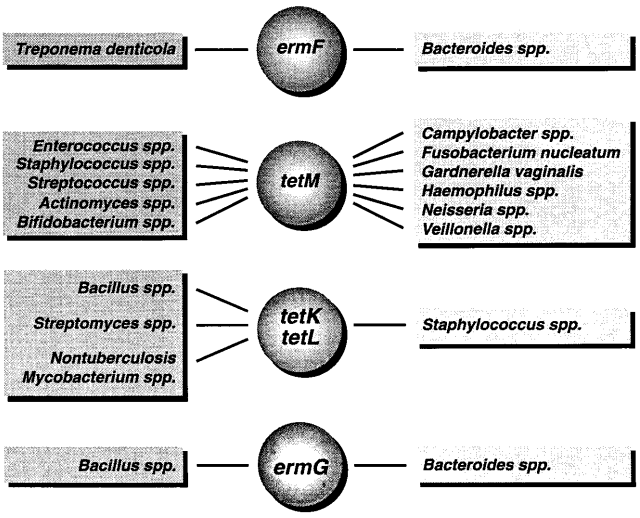

FIG. 1. Evidence that broad-host-range transfer of resistance genes occurs readily in nature (18–20). In each of the cases depicted here, virtually identical copies of the gene in the circle have been found in the species listed in the boxes connected to the circle. Such examples do not prove direct transfer between the species listed but indicate that there is some way for genes to move, whether directly or indirectly, between these genera.

case for *ermG* and *tetM*, this conclusion seems fairly safe. For resistance genes found in the chromosome and not associated with any known conjugal element, however, the mechanism of horizontal transfer is not so certain. It is well established from laboratory experiments that conjugation is capable of mediating very broad host range gene transfers, whereas natural transformation or bacteriophage transduction tend to have more limited ranges. Accordingly, this minireview will focus on conjugal transfer elements, but it is important to keep in mind that examples of broad-host-range transfer events mediated by transformation or transduction may well be found in the future.

HUNTING AS A PACK: INTERACTIONS AMONG GENE TRANSFER ELEMENTS

Two types of broad-host-range conjugal elements have been identified: plasmids and conjugative transposons (Table 1). Whereas plasmids are familiar to most scientists, conjugative transposons are a relatively recent discovery and are not so widely known. Yet, many of the genes included in Fig. 1 (e.g., *tetM*, *tetQ*, *ermF*, and *ermG*) are carried on conjugative transposons. This observation suggests that conjugative transposons are making a significant contribution to the spread of resistance genes, especially among the *Bacteroides* spp. and gram-positive bacteria.

Conjugative transposons are DNA segments, ranging in size from 18 to over 150 kbp, which are normally integrated into the bacterial genome (19). To transfer, they first excise themselves to form a nonreplicating circular intermediate. The circular intermediate is then transferred by conjugation to a recipient, where it integrates into the recipient's genome. Integration is mediated by an integrase carried on the conjugative transposon. Conjugative transposons differ from conventional transposons in that they have a circular intermediate, transfer by conjugation, and do not create a target site duplication when they integrate.

First discovered in gram-positive cocci and *Bacteroides* spp., conjugative transposons are now being found in a variety of bacterial genera including the enteric bacteria (19, 20), and it is likely that they are as widespread and diverse as self-transmissible plasmids. The relatively recent discovery of conjugative transposons, at a time when conjugal transfer had become synonymous with plasmid transfer, raises the question of whether there are still other types of conjugal gene transfer elements that have yet to be identified.

Plasmids and conjugative transposons are proving to be very interactive gene transfer elements (Fig. 2). It has been known for a long time that self-transmissible plasmids, such as the IncP plasmid illustrated in Fig. 2, can mobilize other plasmids residing in the same donor strain, either by providing the mating apparatus through which the other plasmid moves (*trans* mobilization) or by forming a cointegrate with the other plasmid (*cis* mobilization). Recent studies have shown that plasmid mobilization can occur even if the mobilizing plasmid and the plasmid being mobilized are in two different bacterial cells (1, 4, 21). This type of mobilization, in which a donor strain carrying a self-transmissible plasmid acquires a second plasmid from a recipient strain, has been called retrotransfer. Retrotransfer appears to occur in two steps: the self-transmissible plasmid first moves from the donor to the recipient and then mobilizes the plasmid in the recipient back to the donor (5). Since the ability of a self-transmissible plasmid to foster the acquisition of new plasmids by its bacterial host could well confer a selective advantage on the donor bacterium, retrotransfer may have played an important role in the evolution of plasmid mobilization systems.

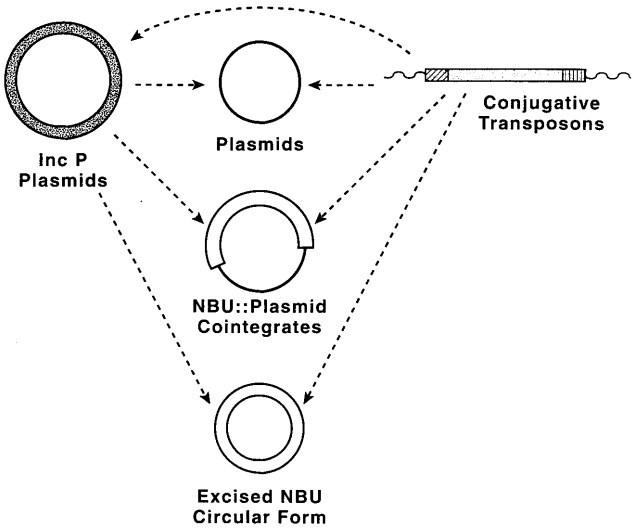

FIG. 2. Interactions between conjugal elements (19, 20). Dashed lines indicate that the element where the line originates can mobilize the element to which the arrowhead points. NBU::plasmid cointegrates are initially nonmobilizable plasmids that have acquired an NBU or an NBU-like element. The conjugative transposons depicted here are the *Bacteroides* conjugative transposons. Tn*916*-type conjugative transposons can mobilize some plasmids but have not been tested for the ability to mobilize the range of elements depicted here.

Conjugative transposons can also mobilize coresident plasmids (Fig. 2). *Bacteroides* conjugative transposons are capable of both *cis* and *trans* mobilization, but the gram-positive conjugative transposon Tn*916* appears to be capable of only *trans* mobilization (19). No attempt has yet been made to determine whether conjugative transposons can mediate retrotransfer. Some *Bacteroides* plasmids are mobilized both by IncP plasmids and conjugative transposons, and at least one IncP plasmid, R751, is mobilized by *Bacteroides* conjugative transposons (19). Mobilization involving elements from completely different phylogenetic groups of bacteria is surprising because these elements presumably did not have much contact with each other during evolution. Such transphylum cooperation underscores the wide scope of interactive potential found in gene transfer elements.

Not all mobilizable elements are plasmids. Some small integrated elements called NBUs (nonreplicating *Bacteroides* units) are excised and mobilized by conjugative transposons (19). NBUs are 10 to 12 kb in size and integrate by a mechanism most similar to that of lambdoid phages. To transfer, they excise from the chromosome to form a covalently closed nonreplicating circle, which is mobilized in *trans* by the conjugative transposon into the recipient, where the NBU integrates once again into the recipient's genome. NBUs have so far been found only in *Bacteroides* strains, but they are capable of transferring to and integrating into the genome of *E. coli*. Most of the NBUs characterized to date have been cryptic, but some carry antibiotic resistance genes (22). It remains to be seen whether mobilizable integrated elements like the NBUs are found in other phylogenetic groups of bacteria.

Interactions between gene transfer elements have been most intensively studied in clinical isolates, but recent studies of gene transfer elements in soil and marine bacteria have shown that mobilizing plasmids and mobilizable plasmids are quite common in environmental settings. Mobilizing plasmids can be

isolated readily by performing a "triparental" mating between *E. coli* carrying a mobilizable IncQ plasmid, a plasmid-less recipient, and mixtures of indigenous soil or marine bacteria (24). They can also be detected in environmental samples by PCR amplification of known incompatibility groups (12). Results of such studies suggest that there are selective pressures in nature that not only favor the maintenance of plasmids and other gene transfer agents but that also promote their interactions with each other.

SETTLING IN: STABLE MAINTENANCE OF RESISTANCE GENES AND RESISTANCE TRANSFER ELEMENTS IN THE ABSENCE OF ANTIBIOTIC SELECTION

The widespread existence of plasmids in natural isolates and their apparent stability, even where antibiotics are not present (12, 24, 25), argue against the widely held belief that plasmids and other gene transfer elements are readily lost in the absence of selection. Moreover, the abundance of antibiotic-resistant strains in environmental settings where the bacteria presumably do not come into contact with antibiotics (4, 7, 16) suggests that resistance genes can also be stably maintained in the absence of antibiotic selection. Much has been written about the fact that plasmids carrying multiple resistance genes can be held in a bacterial strain by selection for any one of the resistance genes on the plasmid, but remarkably little is known about the reasons for the stability of gene transfer elements and resistance genes in the absence of any known selection pressure. Either there are as yet unidentified selection pressures or the inherent stability of naturally occurring resistance genes and resistance gene transfer elements is sufficient to maintain them in the absence of selection.

One way that a plasmid carrying a resistance gene can be maintained stably in the absence of antibiotic selection is to carry other genes that confer a different kind of selective advantage (1). For example, plasmids can encode enzymes that increase the colonization proficiency of the bacterium, and selection for these other traits provides a coselection for maintenance of resistance genes carried on the plasmid. The existence of integrons (13) suggests that this survival strategy is indeed important for plasmid maintenance. Integrons, which are usually found on transposons, specialize in creating clusters of genes (13). The integron provides an integration site for incoming gene cassettes plus an integrase that mediates orientation-specific integration of these genes and a promoter that ensures expression of the operon created by integrase action. In addition to antibiotic resistance genes, integron-generated operons frequently contain genes that confer resistance to heavy metals. Thus, heavy-metal pollution or the mercury in mercury amalgams such as dental fillings could provide the selective pressure that maintains the plasmid and its gene cluster even in the absence of antibiotic selection. This may explain why Summers and coworkers (23) found a higher number of antibiotic-resistant *E. coli* strains in the feces of people and animals with dental fillings that contain mercury. It is even possible that the conjugal transfer machinery itself confers a selective advantage not just because of its ability to transfer DNA but also because it can function as an adhesin (3).

Less is known about the stability of conjugative transposons, although they appear to be quite stable in most hosts (19). The fact that they integrate rather than replicate autonomously may contribute to their maintenance. Even in hosts where they are unstable initially, they may be able to fix themselves by undergoing DNA rearrangements.

Mutations that increase stability are known to occur in the case of plasmids. Lenski and coworkers (14) have shown that repeated subculture of a plasmid-containing strain under selective conditions eventually gave rise to a variant of the plasmid that was much more stable in the absence of selection than the original form of the plasmid. Their findings suggest that exposure of a bacterium with a newly acquired plasmid or conjugative transposon to antibiotic concentrations high enough to be slightly selective but low enough to allow bacteria to replicate could foster adaptive mutations that have the effect of fixing the element in its new host. Similarly, any resistance genes carried on the plasmid or conjugative transposon would have the chance during this period of selective pressure to increase their expression levels (6) or to adapt to a better fit with their new host (14). Still another route to stability is exemplified by plasmids that kill daughter cells which do not inherit a copy of the plasmid (1).

Conjugal transfer itself can contribute to stable maintenance of antibiotic resistance genes in a bacterial population by continually reseeding members of a population that have lost a resistance gene transfer element. It is noteworthy that exposure to low concentrations of tetracycline induces transfer of *Bacteroides* conjugative transposons, which generally carry tetracycline resistance genes (19). Exposure of a bacterial population to tetracycline would thus have the effect of rapidly increasing the number of members that harbor the conjugative transposon. The fact that some conjugal elements have a very broad host range makes possible another variation on this theme: temporary escape of a gene transfer element into another species or genus. Thus, for example, a conjugal element that is not stably maintained in a pathogenic bacterium without selection could survive periods of no selection by moving into a human commensal or soil bacterium, where it is available for later reacquisition by the pathogenic species.

WHERE DO WE GO FROM HERE?

Plasmids and conjugative transposons have developed an impressive capacity for interactions with each other and with other gene transfer elements. These interactions increase not only the frequency of resistance gene transfer but also the range of transfer, because they allow a mobilizing element to move another plasmid or integrated element into a recipient where the mobilizing element itself is lost. Why this capacity to interact has evolved and is so widespread remains unclear. The interacting systems are so diverse that there must have been strong selection for interactions between gene transfer elements even in the preantibiotic era. It would be useful to know what environmental pressures promoted the development and survival of interactive gene transfer elements because such information might suggest novel strategies for countering the spread of resistance genes. Also, the widespread existence of interactions between different elements raises questions about the appropriateness of models for resistance gene ecology that are based on movement of a single plasmid.

More attention needs to be paid to the stability of resistance genes and the conjugal elements that carry them. The fact that stability seems to be the rule rather than the exception in natural isolates suggests that the best place to stop resistance is before it starts, by preventing the acquisition of resistance genes in the first place. The fact that a resistance transfer element, which is initially somewhat unstable, can become more stable if it is given time to adapt suggests that long-term exposure to low antibiotic levels is the condition most likely to foster stable maintenance of resistance genes because it gives incoming elements and resistance genes a chance to adapt to their new host. By contrast, short-term exposure to levels of

antibiotics high enough to kill bacteria or prevent their growth entirely is much less likely to foster resistance.

It is time to take a new look at the spread of antibiotic resistance genes. We must not base all of our notions about resistance genes on laboratory studies of a few strains of *E. coli*—or even on epidemiological studies that only follow *E. coli* and its close relatives. More information is needed about resistance gene movement and the stability of resistance genes in the gram-positive pathogens and in the major members of the intestinal microbiota, such as gram-positive anaerobes and *Bacteroides* spp. In addition, we should broaden the focus of epidemiological studies to include nonclinical isolates. Many people assume that antibiotic-resistant strains are arising primarily in hospitals or clinics, but is this really true? The widespread use of antibiotics by dermatologists, dentists, and farmers could be just as great a force for resistance development as hospitals. Finally, we need to determine whether there are environmental selection pressures, other than antibiotic use, that contribute to the spread and maintenance of resistance genes and that explain the high level of resistance in areas where antibiotics appear not to be present.

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