Antibiotic Resistance Plasmids in Bacteria

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Antibiotic resistance plasmids are bacterial extrachromosomal elements (plasmids) that carry genes conferring resistance to one or more antibiotics. They are notorious for their ability to transfer conjugatively between bacterial species and are significantly involved in the emergence and dissemination of multiple drug resistance.

Secondary article

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Introduction

The discovery of antibiotics and their clinical potential in treating bacterial infections in humans in the 1930s and 1940s represented an exciting and extremely important milestone in the field of medicine. Countless numbers of people are alive today or significantly extended their lives as a result of treatment with these compounds. Unfortunately, as each new antimicrobial agent was introduced, resistant strains began to appear within a few years. One of the first and classic examples of this phenomenon involved the use of sulfonamides, which were effective in the treatment of dysentery in Japan at the end of World War II. By 1952 more than 80% of *Shigella* strains involved in dysentery outbreaks were resistant. The early 1950s brought the introduction of new drugs, such as streptomycin, chloramphenicol and tetracycline, which were effective against dysentery, but only for a few years. A significant observation was that some strains involved in the same epidemic were completely sensitive, while others were resistant to all four drugs. Moreover, some patients carried strains of *Escherichia coli* that were resistant to all four compounds. It was eventually found that the multiple-resistance traits of *E. coli* could be passed to a sensitive *Shigella* strain in a linked fashion by simply mixing the two strains together in a suitable culture medium (Watanabe, 1963). Although not clearly understood at the molecular level at the time, this represented an important demonstration that antibiotic resistance determinants could reside on mobile genetic elements (R-factors) able to transfer from one bacterial strain (or species) to another. Investigation of the phenomenon over the succeeding years has revealed the extensive role played by agents, called plasmids, and related components, known as transposons and integrons. The general nature and properties of these entities and their contribution to the general problem of antibiotic resistance are described below.

General Nature of Antibiotics and Bacterial Resistance

Antibiotics exhibit their selective toxicity in a variety of ways: some are effective over a broad spectrum of bacteria, whereas others work efficiently only against certain groups or species. Therapeutically useful compounds are usually the products of specific strains of bacteria or fungi and represent relatively few of what are probably thousands of different antibacterial compounds, more broadly known as bacteriocins. The production of bacteriocins is extremely widespread and doubtless has played an important role in competition within the bacterial world for millions of years. Most bacteriocins are very specific in their mode of action and may target only one or a few species of bacteria, whereas those that exhibit a broad host range have potential value as therapeutic antibiotic agents. Some, such as the bacteriocin nisin, have been used as food preservatives. During the microbial competition ongoing in soil, on plants or within animal systems over the years, the development of defensive mechanisms (i.e. resistance) occurred concurrently. Indeed, the producers of bacteriocins/antibiotics require a means of protection from their own weapons.

Resistance mechanisms vary significantly depending on the particular antibiotic. Many are highly evolved and relate to such processes as enzymatic modification/inactivation of the drug, modification of the drug's target, or export of the agent from the cell. In only some cases does clinical resistance relate to recent mutations in the drug target (e.g. ribonucleic acid (RNA) polymerase in the case of rifampin and deoxyribonucleic acid (DNA) gyrase in the case of the quinolones), and even here, this is not always the case. In recent years, individual genes conferring multiple drug resistance have been identified and found to encode efflux systems consisting of membrane proteins able to pump a broad spectrum of chemical agents out of the cell (Edgar and Bibi, 1997).

It is likely that some resistance genes of clinical significance originated from those found in the antibiotic-producing organisms, whereas others may have evolved from certain 'housekeeping' genes (Davies, 1994). The notion that many antibiotic resistance genes have been around long before the medical usage of the corresponding drugs is supported by the identification of resistant isolates from humans in remote populations never previously treated (Mare, 1968). From this perspective it should not be surprising that the widespread use of antibiotics in humans and animals has resulted in only a few decades in a situation where multiple resistance has become the significant problem it now represents. In recent years it has reached a point where vancomycin has become the 'last resort' antibiotic in the case of certain human pathogens. And now there is great concern that resistance to this agent is emerging rapidly, especially among the enterococci. This has brought about a sense of urgency within the pharmaceutical industry in its efforts to develop new agents, a few of which have just come on the market or will soon be available.

Plasmids as Carriers of Antibiotic Resistance Genes

Plasmids are extrachromosomal genetic elements commonly found in bacteria. These DNA molecules are relatively small compared to the bacterial chromosome and range in size from a few kilobases to well over 150 kb; they are generally circular but examples of linear plasmids have been reported (e.g. in certain species of *Borrelia* and *Streptomyces*). Plasmids commonly carry genes useful to the bacterial host only under atypical conditions, and antibiotic resistance genes fit nicely into this category. Indeed, plasmids often carry drug resistance genes and can sometimes confer resistance to a number of different drugs. Similarly, plasmids can confer resistance to metal ions (e.g. Hg^{2+}), certain antiseptic/disinfectant compounds (e.g. chlorhexidine or quaternary ammonium compounds) and even ultraviolet light.

Plasmids vary widely in their ability to maintain themselves in different species of bacteria. Those able to replicate in a given host can frequently be categorized into different families, called incompatibility groups, based on whether they can coexist independently in the same cell. Members of the same incompatibility group utilize similar replication and maintenance machinery and therefore compete with each other in such a way that only one member survives without selection. Some plasmids have a broad host range in which they are able to replicate (e.g. RSF1010 in Gram-negative bacteria), and a few are even known to replicate in both Gram-positive and Gram-negative bacteria (e.g. pMV158).

While many plasmids carry one or more resistance determinants, some carry genes that contribute to bacterial virulence; and there are many others, the so-called 'cryptic' plasmids, that carry genes of unknown function. In the case of the Gram-negative *E. coli* or the Gram-positive *Enterococcus faecalis*, intestinal human isolates simultaneously carrying up to five or six different plasmids are not unusual; most of these fall into the cryptic category.

A given plasmid characteristically maintains a specific copy number, with the larger plasmids (e.g. greater than 25 kb) usually at 1–2 copies per host chromosome and the smaller (e.g. 5–10 kb or less) often present in multiple (e.g. 20–30) copies. In the case of *Col*E1 (a 6-kb element in *E. coli* determining a bacteriocin) and related plasmids, exposure to the bacteriostatic drug chloramphenicol allows continued replication of plasmid DNA, resulting in several thousand copies per cell (i.e. over 100-fold higher than its normal copy number), while cell division and chromosomal replication are prevented (Clewell, 1972). Such cells could provide a significant reservoir of plasmid DNA that might be 'picked up' by other bacteria (see below). From an evolutionary perspective, this may represent an effort by the plasmid to enhance its probability of survival under conditions hostile to its host.

Another type of 'amplification' relates to the selection of repeated copies of a resistance determinant on a single plasmid, on the basis that increased dosage of the determinant can result in increased resistance. A good example of this is the case for the small *Ent. faecalis* plasmid pAMα1, which carries a tetracycline resistance determinant flanked by two 380-bp direct repeats. Growth of cells in the presence of tetracycline gives rise to plasmid DNA that may have as many as 9–10 tandemly repeated copies of the resistance determinant (Yagi and Clewell, 1977). The process is dependent initially on homologous recombination between the flanking repeat sequences, a process that could allow excision of the resistance determinant (*tet*) followed by insertion into one of the repeat sequences of a coresident intact element. An alternative process would involve recombination between repeat sequences on opposite sides of *tet* within replicated segments of a partially replicated plasmid molecule. Such a process would lead to one of the daughter plasmids having a duplication of *tet* while the other has lost (deleted) the determinant. Once there are at least two tandem copies of the larger resistance-carrying segment, the recombination potential increases because of the significant size increase of repeated homologous DNA. Subsequent growth in the absence of drug results in a return to the initial state by excision and segregation of the extra copies of *tet* that are no longer needed. Similar examples of this have been reported in the case of certain large multiple resistance plasmids in Gram-negative bacteria (Rownd *et al*., 1979).

Conjugation and the Movement of Plasmids Between Bacteria

Bacterial traits associated with plasmids are often viewed as having been acquired relatively recently by horizontal transfer – that is, by the acquisition of DNA from another bacterial cell. Although plasmid DNA can move from one cell to another by the phenomena of transformation (i.e. release of DNA by lysis and uptake by competent recipients) or by transduction (i.e. with the help of a bacterial virus), the most highly evolved mechanism may relate to the ability of certain elements to move by cell-to-cell contact. This phenomenon is called conjugation, and plasmids able to transfer a copy of themselves generally carry a special set of genes that direct this process. Not all plasmids carry such genes, although many have determinants that allow them to be efficiently mobilized by coresident conjugative plasmids. Other plasmids lack even these determinants but may still be mobilized by conjugative elements by the formation of transient or stable fusions, called cointegrates, that occur as part of a homologous or specifically directed recombination process. Certain cointegrates may have an added advantage whereby one of the component plasmids may be capable of replicating in a bacterial host/species in which its 'associated' plasmid could not exist independently.

The conjugative or mating event has in some cases been shown to involve the acquisition of a single-stranded nick at a site called the origin of transfer (*oriT*); and this is followed by the transfer of a single strand of plasmid DNA into the recipient bacterium. The complementary strand is synthesized in the recipient and is also replaced in the donor – to some extent making use of host replication machinery. The net result is that the donor cell has passed only a copy of its plasmid to the recipient, which can then pass additional copies on to new recipients. Under selective conditions, such as when an antibiotic resistance determinant is located on the plasmid and when the bacterial host is exposed to the related antibiotic (e.g. during treatment for an infection), there may be a significant population increase of the plasmid-bearing organism (not necessarily the disease-causing organism but others in the intestine), which in turn provides a large donor potential for further dissemination of the plasmid.

Some resistance plasmids transfer quite efficiently (e.g. approaching 100% under optimum conditions in the laboratory) and exhibit a broad host range. Thus some plasmids, such as RP4 in Gram-negative bacteria and pAMβ1 in Gram-positive bacteria, can transfer into a variety of different species, including between nonpathogens and pathogens. Considering the large number of different bacteria (estimated at as many as a thousand types) located in the mammalian intestine, it is easy to envision how resistant organisms of all kinds might readily emerge.

The plasmid-borne genes that confer the ability to conjugate include those that facilitate the generation of cell-to-cell contact (**Figure 1**). In Gram-negative bacteria, the use of sex pili or fimbriae extending from the cell surface is well known. Such plasmid-encoded structures serve the donor by adhering to potential recipients and generating the close contact necessary prior to the transfer of DNA. Some pilus structures, such as in the case of the classic sex factor F in *E. coli*, are relatively long and, after binding recipients at their tip, appear to retract – pulling the

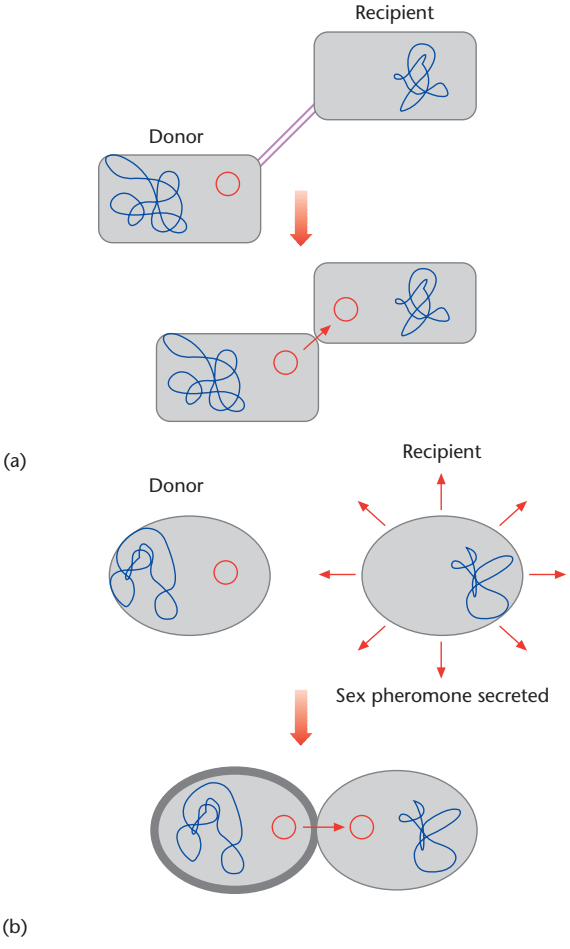

Figure 1 The generation of cell-to-cell contact involved in conjugation. (a) An *Escherichia coli* cell with its plasmid-encoded pilus structure binding to the recipient (plasmid-free) cell and retracting in order to generate direct contact between the two cells. (b) An *Enterococcus faecalis* donor cell undergoing a plasmid-encoded mating response to a peptide sex pheromone secreted by a recipient cell. The donors synthesize 'aggregation substance' that coats the surface and facilitates adherence to recipients upon random collision. Once a copy of the plasmid is acquired, the resulting transconjugant cell shuts down or masks the endogenous pheromone and becomes a potential donor. Interestingly, transconjugants continue to produce other peptide pheromones able to induce a mating response by donors carrying different families of conjugative plasmids. A plasmid-free strain can actually make up to six different pheromones, and probably many more.

recipient into direct membrane contact (Firth *et al*., 1996). Other plasmids facilitate the initiation of cell contact via the synthesis of a pheromone-induced 'fuzzy' microfibrillar surface material. An example is the 'aggregation substance' that is encoded by certain conjugative plasmids in *Ent. faecalis* (Clewell, 1999).

Insertion Sequences and Transposons

Resistance genes are very commonly located on segments of DNA that are able to 'jump' from one location to another by a process known as transposition, and the related mobile DNA sequences are called transposons (Craig, 1996). Transposons are actually more evolved forms of smaller movable DNA segments called insertion sequences or IS sequences. The latter tend to be of the order of 700–1500 bp in size and determine a specific recombinase (transposase) that facilitates the movement. The ends of an insertion sequence usually contain inverted repeat sequences corresponding roughly to 20–40 nucleotides which are recognized by the transposase. Typically these elements may move from one place to another (e.g. from one site on the chromosome to another, or from the chromosome to a plasmid, etc.) at frequencies of the order of 10^{-6}–10^{-4} per cell division. A characteristic feature is the ability to move in bacterial hosts that are defective in homologous recombination (e.g. in the absence of the RecA protein in the case of *E. coli*).

Target sites for insertion sequences usually have little specificity; thus insertions can occur almost anywhere in a DNA molecule. Genetic mutations occur commonly via insertions into bacterial genes, and these elements probably play a major role in evolution. Some insertion sequences have outwardly firing promoters that may influence expression of adjacent genes. In *E. coli*, insertion sequences are designated IS*1*, IS*2*, IS*3* etc.; many are present on the chromosome in multiple copies. Because they may at the same time be located on a resident plasmid, they can serve as portable regions of homology for homologous recombination. Indeed, the sex factor F carries such elements (e.g. IS*2* and two copies of IS*3*), which allow the formation of cointegrate structures with the chromosome; these correspond to Hfr (high-frequency recombination) derivatives because, under the direction of the F plasmid, the entire chromosome can transfer conjugatively to recipients where a 'high frequency' of recombination of chromosomal genes can then occur. Recombination of this nature between different resistance plasmids as well as other replicons is believed to be common, as insertion sequences are ubiquitous in the bacterial world.

With regard to the transpositional properties of insertion sequences, a newly inserted DNA segment exhibits a duplication of the target sequence which appears on both sides of the element. The repeats have the same orientation, and their length is characteristic of the particular element (e.g. five nucleotides in the case of IS*2*). The movement of some insertion elements from one plasmid to another may result in a stable cointegrate; in other cases this does not happen. In some cases a cointegrate structure may correspond to an intermediate in the transposition process, with copies of the element located at the two plasmid junctions; in such cases the two replicons are eventually resolved. The process is considered replicative because, even though the target element receives a copy, the transposon remains at its original location.

Transposons move essentially the same way insertion sequences move and in some cases simply represent a resistance determinant that is flanked by two insertion elements. The sequences can be in either direct or inverted orientation with respect to each other, and they are able to supply the transposition functions necessary to move the complete unit. The outside ends work together to move the entire segment as a composite element. Some examples of these so-called 'composite transposons' are Tn*5* (determines kanamycin resistance) which is flanked by two IS*50* elements, Tn*10* (determines tetracycline resistance), which is flanked by IS*10* sequences, and Tn*4001* (encodes resistance to gentamicin and kanamycin), which is flanked by IS*256* sequences.

Some transposons represent a more independent unit and are not simply segments of DNA separated by insertion elements. Their mechanisms of transposition are similar to those of the composite elements, in that they provide their own transposition genes, although in some cases movement may be a more complex process. Some examples of these are Tn*3* (encodes ampicillin resistance), Tn*7* (encodes resistance to trimethoprim and streptomycin), and Tn*917* (encodes resistance to erythromycin) (**Figure 2**). In some cases exposure of cells to the agent to which a resident transposon encodes resistance can

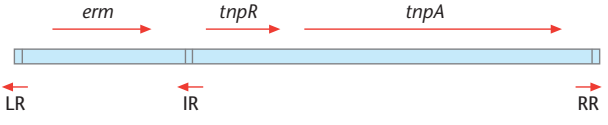

Figure 2 Diagram of the erythromycin-resistance transposon Tn*917* originally identified in *Enterococcus faecalis*. The element is a little over 5000 bp long and is bounded by 38-bp inverted repeats (LR, left repeat; RR, right repeat) indicated by the short arrows at the left and right ends. IR (internal repeat) is also a 38-bp repeat which conceivably could work together with RR to move part of the element without the erythromycin resistance gene *erm*, although this has not actually been demonstrated. *tnpA* encodes the transposase and *tnpR* encodes a 'resolvase' that acts subsequent to the transposase in 'resolving' a cointegrate structure that represents an intermediate in the transposition process. The resistance determinant of Tn*917* is inducible, in that a subinhibitory concentration of erythromycin greatly enhances the level of the *erm* product by an increase in transcription. This can also have an effect downstream by increasing expression of the transposition genes, which can lead to an increase in the frequency of movement of the element.

enhance the transposition frequency. Examples of this are Tn*917* and Tn*501* (encodes resistance to Hg^{2+}). The basis of this relates to the fact that the resistance determinants are themselves inducible, and there is some transcriptional readthrough into transposase determinants.

Insofar as transpositional elements are constantly moving around, it is not surprising that resistance genes tend to accumulate on plasmids. While movement from one site to another is generally random, a plasmid that has picked up such a sequence may be capable of rapidly moving to another cell and beyond – thus rapidly amplifying its population. In some cases there may be direct selection for a resistance transposon that has moved, for example, from the low-copy chromosome, to a high-copy number plasmid, as the cells may have an increased growth advantage due to an increased dosage of the resistance determinant.

Conjugative Transposons

Some transposons are capable of intercellular transposition and are referred to as conjugative transposons. Exemplified by Tn*916* (encodes tetracycline resistance) and Tn*1545* (encodes resistance to tetracycline, erythromycin and kanamycin), these elements are particularly common in the enterococci and streptococci; however, they have a broad host range that extends even to Gram-negative bacteria (Clewell *et al.*, 1995). Tn*916* and related elements, for example, have been found to move into or occur naturally in over 50 different bacterial species. Such elements are usually, but not always, located on the bacterial chromosome; and in some species such as *Streptococcus pneumoniae* and *Streptococcus pyogenes*, they appear more likely to be involved in acquired antibiotic resistance than plasmids.

Movement of conjugative transposons involves an excision event that gives rise to a circular intermediate which is incapable of autonomous replication. Whereas the intermediate is capable of inserting at numerous sites elsewhere in the same cell, a battery of transposon-encoded conjugation functions allows them to transfer into another cell, much like a plasmid, where they are believed to take on again a circular state prior to insertion into recipient DNA. The 18-kb Tn*916* has been genetically analysed and completely sequenced; and a specific site has been identified that serves as the origin of conjugative transfer. Tn*916* and Tn*1545* have been shown to be flanked by 6-bp 'coupling sequences' that are not identical; however, it is believed that these sequences participate in the recombinational loop-out which leaves one coupling sequence joining the two ends of the transposon in the intermediate. The specific nature of the coupling sequences flanking an insert greatly influences the frequency of excision, and this ultimately affects the frequency of conjugative transposition. Transfer frequencies can vary from 10^{-9} to 10^{-4}, depending on the coupling sequences.

Conjugative transposons are also known to occur in *Bacteroides* species (Gram-negative anaerobes) but they exhibit a much higher degree of site specificity. Some are able to transfer between *Bacteroides* and *E. coli*; however, they are not yet known to have a particularly broad host range. Interestingly, certain tetracycline resistance elements of this nature transfer at frequencies 1000-fold greater if the cells are exposed to a low level of tetracycline.

Integrons

Nucleotide sequence analyses have revealed that many drug resistance determinants correspond to 'cassettes' frequently located in tandem on a plasmid and in some cases within a transposon. Such structures are components of what are now referred to as integrons (Recchia and Hall, 1997). An integron contains a determinant for a specific integrase (recombinase), designated *intI*, that facilitates the capture of cassettes which become tandemly associated. A cassette contains a promoterless gene with a recombination site known as a '59-base element' (59-be) located at its 3′ end (**Figure 3**). The size of the 59-be can vary depending on the cassette and can in some cases be over 100 bases long; but in all cases the outer ~25 nucleotides correspond to inverted repeat sequences. The integrase facilitates the movement of cassettes by catalysing excision via recombination between flanking recombination sites, resulting in a circular but not replicative cassette containing the determinant and the 59-be. Insertion (integration) generally occurs at a recombination site called *attI* located immediately adjacent to *intI*. The *intI* determinant is oriented opposite to the direction of its associated cassettes, but

Figure 3 Structure of an integron. The arrows indicate the relative orientation of the various components. P is the promoter that fires backwards from a site within the 5′ end of the integrase determinant *intI*. (*intI* has its own promoter (the circle at the beginning of the arrow) upstream.) *attI* is the site where new cassettes enter and become part of the integron. Cassettes move by excision via a 'loop-out' involving flanking recombination sequences (i.e. 59-base element (59-be) sequences). The nonreplicating circular element containing one 59-be then enters at the *attI* sequence. Expression is greatest at the head of the line because it is closest to the promoter P; and exposure to a given antibiotic will select for movement of the appropriate cassette to this position.

within its 5′ end is a strong promoter that fires 'backwards' into the adjacent resistance genes. Thus, newly acquired cassettes are said to enter 'up front' and, because of their position closest to the promoter, can express at a 'maximum' level. Transcription of cassettes further downstream may occur to lesser degrees, depending on the distance from the promoter. One can easily visualize the selection for resistance determinants that leapfrog from a distal site to the most expressive site adjacent to the promoter, depending on environmental conditions.

A given integron may carry determinants with different codon usage, suggesting they have widely diverse origins. Most elements that have been characterized have been associated with Gram-negative bacteria; however, there are recent reports of such structures in Gram-positives. Interestingly, a segment of the *Vibrio cholerae* genome has been found to consist of arrays of genes separated by short inverted repeat sequences of 123–126 bp. Designated as *V. cholerae* repetitive sequences (VCRs), as many as 100 copies of such sequences are present in the genome. The outer ends of a VCR have been shown to relate to a 59-be; and additional resemblances to integron elements have been identified (Barker *et al*., 1994). It is likely, therefore, that integrons are ubiquitous and play an important evolutionary role in not only the emergence of multiple drug resistance but a variety of other traits as well.

Concluding Remarks

Analyses of plasmids and the genetic information they carry have revealed important insights into how and why we have reached the antibiotic resistance problem we now confront. At the same time, information gained from such investigations illustrates important aspects of microbial evolution. The potential for recombination of genes from widely diverse bacterial populations is vast; and it appears that it is simply a matter of time before a given bacterial species acquires the genetic means to survive under conditions that might otherwise inhibit growth. It is clear that the extensive use of antibiotics by humans has resulted in the emergence of clinically resistant bacteria, and the heavy utilization of some of these agents in agriculturally related practices (e.g. in animal feeds, commercial fisheries, plant and tree sprays, etc.) is also a major factor. The result is that cases are increasing where certain human infections simply cannot be treated (e.g. certain enterococcal-related cases of infective endocarditis). New drugs such as synercid and the oxazolidinones should help in dealing with some of these infections; but the time during which these agents remain effective will be limited, as new resistant strains emerge to meet the challenge.

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