Inactivation of Antibiotics and the Dissemination of Resistance Genes

Julian Davies

The emergence of multidrug-resistant bacteria is a phenomenon of concern to the clinician and the pharmaceutical industry, as it is the major cause of failure in the treatment of infectious diseases. The most common mechanism of resistance in pathogenic bacteria to antibiotics of the aminoglycoside, β-lactam (penicillins and cephalosporins), and chloramphenicol types involves the enzymic inactivation of the antibiotic by hydrolysis or by formation of inactive derivatives. Such resistance determinants most probably were acquired by pathogenic bacteria from a pool of resistance genes in other microbial genera, including antibiotic-producing organisms. The resistance gene sequences were subsequently integrated by site-specific recombination into several classes of naturally occurring gene expression cassettes (typically "integrons") and disseminated within the microbial population by a variety of gene transfer mechanisms. Although bacterial conjugation once was believed to be restricted in host range, it now appears that this mechanism of transfer permits genetic exchange between many different bacterial genera in nature.

When antibiotics were first introduced in the 1950s for the treatment of common microbial infections, the bacterial geneticists of the day suggested that the development of antibiotic resistance during therapy was unlikely because the frequency of mutation to resistance in bacteria was too low. More to the point, it was unsuspected that in nature bacteria might collect and exchange genetic information with extraordinary facility and lack of species specificity, thus permitting antibiotic resistance determinants of a variety of different biochemical mechanisms already present in the environment to be "picked up" and passed on from one microbe to another.

In 1940, several years before the introduction of penicillin into clinical practice, Abraham and Chain (*1*) identified a bacterial enzyme that catalyzed the hydrolysis of the β-lactam ring of the antibiotic and eliminated its antibacterial activity, and they noted that this enzyme might interfere with penicillin therapy. Their prediction has been realized to an extent that no one could have anticipated: the enzyme penicillinase, and its many isozymes, has plagued the β-lactam antibiotics ever since and is the single most important consideration in resistance to this class of antibiotics. It must be said, in defense of the microbiologists and physicians of the day, that no one could have anticipated the extent to which antibiotics would be made and used worldwide during the past half-century [1994 marks the 50th anniversary of the discovery of streptomycin by Waksman and Schatz (*2*)]. This has not only led to a catastrophic situation for microbes but at the same time has created enormous pressure for the selection of antibiotic resistance traits. In this brief review, I propose to survey the mechanisms by which antibiotics may be altered (detoxified) in resistant strains and the means by which these evolved resistance processes have been disseminated throughout the microbial population.

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Mutation and Gene Acquisition

Most of the early studies of antibiotic resistance involved laboratory experiments with Enterobacteriaceae such as *Escherichia coli* or *Salmonella typhimurium*, and although spontaneous mutants resistant to streptomycin (the most studied antibiotic at the time) could be isolated, they were found to occur at frequencies of 10^{-9} or less per bacterial generation. Under these circumstances the development of antibiotic-resistant strains during therapy was unlikely to be a serious clinical problem. These studies could not have anticipated the wide variety of resistance mechanisms nor the possibility that genes encoding resistance to antibiotics would be carried by autonomously replicating transferable elements called resistance plasmids (R plasmids).

Among the mechanisms identified (Table 1), enzymic inactivation of antibiotics is one of the most common biochemical processes that engenders resistance to a wide variety of antibiotic structural types in bacteria (*3*, *4*). In retrospect, it is surprising that inactivation mechanisms have been so important in the determination of antibiotic resistance because a priori one would not expect such processes to arise by mutation: How could a few simple amino acid changes in an existing (and often essential) bacterial enzyme permit the enzyme to catalyze the structural modification of a completely different substrate? Determinants for the enzymic inactivation of antibiotics in clinical isolates could have arisen in most cases only by the inheritance of exogenous functions (genes). This is not to say that the process of mutation is not an influential factor in the development and evolution of clinically important antibiotic resistance. As discussed below, once established in a pathogen, genes encoding enzymes that catalyze covalent modifications of therapeutic agents can undergo mutations that remodel the active site of the enzyme, changing the spectrum of antibiotic substrates that may be modified. The disturbing appearance of bacterial strains resistant to the fluoroquinolone antimicrobials is also due to spontaneous mutations in bacterial genes (*5*).

Parenthetically, mutational alteration of antibiotic target sites is one of the reasons for the recent upsurge in multidrug-resistant mycobacterial infections. In the slow-growing *Mycobacterium tuberculosis*, mutants resistant to streptomycin appear more frequently than they do in *E. coli*. Streptomy-

Table 1. Transferable antibiotic resistance in bacteria. For a thorough overview of biochemical mechanisms of drug resistance, see Hayes and Wolf (*67*).

Mechanism	Antibiotic
Reduced uptake into cell	Chloramphenicol
Active efflux from cell	Tetracycline
Modification of target to eliminate or reduce binding of antibiotic	β-lactams Erythromycin Lincomycin
Inactivation of antibiotic by enzymic modification:	
Hydrolysis	β-lactams Erythromycin
Derivatization	Aminoglycosides Chloramphenicol Fosfomycin Lincomycin
Sequestration of antibiotic by protein binding	β-lactams Fusidic acid
Metabolic bypass of inhibited reaction	Sulfonamides Trimethoprim
Binding of specific immunity protein to antibiotic	Bleomycin
Overproduction of antibiotic target (titration)	Sulfonamides Trimethoprim

cin acts by inhibiting protein synthesis on the ribosome; mutations leading to resistance result in an altered ribosomal protein S12 or 16S ribosomal RNA (rRNA) such that the ribosome has reduced affinity for the antibiotic. Most fast-growing bacteria have multiple copies of the rRNA genes, and because resistance is genetically recessive to antibiotic sensitivity, only rare mutations in the gene for protein S12 are isolated under normal situations. However, because the slow-growing mycobacteria possess only single copies of the rRNA genes, streptomycin resistance can arise by mutational alteration of either 16S rRNA or ribosomal protein S12. Both types of mutation have been identified in *M. tuberculosis* (6). It is of note that bacteria resistant to streptomycin were isolated about a year after the discovery of the antibiotic (7).

β-lactamases. In the years after the introduction of the β-lactam antibiotics (penicillins and cephalosporins) for the treatment of Gram-negative and Gram-positive infections, there has been a constant tug-of-war between the pharmaceutical industry and the bacterial population: the one to produce a novel β-lactam effective against the current epidemic of resistant bugs in hospitals, and the other to develop resistance to the newest wonder drug. The role of mutation is especially important in the evolution of resistance in this case. In his 1992 review (3), Neu showed the "phylogeny" of development of β-lactam antibiotics in response to the evolution of bacterial resistance to this class of antibiotics. Of the several known mechanisms of resistance to the β-lactam antibiotics (Table 1), the most elusive moving target is hydrolytic inactivation by β-lactamases. A single base change in the gene for a β-lactamase can change the substrate specificity of the enzyme [Table 2 (8)].

Such changes occur frequently, especially in the Enterobacteriaceae, and it is frightening to realize that one single base change in a gene encoding a bacterial β-lactamase may render useless $100 million worth of pharmaceutical research effort. This cycle of natural protein engineering in response to changing antibiotic-selection pressure has been demonstrated, especially for the β-lactamase (penicillinase and cephalosporinase) genes. The parental genes appear to originate from a variety of different (and unknown) sources (9). The β-lactamase families differ by a substantial number of amino acids, as is the case for other antibiotic resistance genes. Sequential expansion of their substrate range to accommodate newly introduced β-lactam antibiotics is a special case and occurs by a series of point mutations at different sites within the gene that change the functional interactions between the enzyme and its β-lactam substrate. Other resistance mechanisms evolve in response to continued β-lactam use. In some cases, increased resistance results from increased expression of the β-lactamase through an up-promoter mutation (10), or chromosomal β-lactamase genes can be overexpressed in highly resistant strains as a result of changes in transcriptional regulation (11). The fact that the β-lactamase genes so readily undergo mutational alterations in substrate recognition could have several explanations, one being that the β-lactamases, like the related proteases, have a single active site that does not require interaction with any cofactors. Other antibiotic-modifying enzymes often have two active binding sites. In laboratory studies, β-lactamases of altered substrate spectrum can be generated by in vitro mutagenesis; such is not the case for the aminoglycoside-modifying enzymes. The pharmacokinetic characteristics of the different classes of antibiotics (for example, dose regimen, active concentration, or route of excretion) also may favor the pathway of mutational alteration in the development of resistance.

As one approach to counteracting the destructive activity of β-lactamases, a series of effective inhibitors of these enzymes has been employed. These inhibitors are structural analogs of β-lactams that are, in most cases, dead-end irreversible inhibitors of the enzyme. Several have been used in combination with a β-lactam antibiotic for the treatment of infections from resistant microbes, for example, the successful combination of amoxicillin (antibiotic) and clavulanic acid (inhibitor). However, the wily microbes are gaining the upper hand once again by producing mutant β-lactamases that not only are capable of hydrolyzing the antibiotic but concomitantly become refractory to inhibition (12). The vicious cycle continues! Extended-spectrum cephalosporins and related β-lactam and carbapenem antibiotics are being countered by natural genetic engineering of the appropriate extended-spectrum β-lactamases (and other resistance mechanisms).

One should not finish this brief discussion of resistance to β-lactam antibiotics without mentioning that various combinations of resistance mechanisms may be found in the same bacterial strain in hospital isolates. A resistant strain may have a target with reduced affinity for the drug because of an altered penicillin-binding protein (13), a mechanism that reduces antibiotic uptake, and a mechanism for inactivating the drug. Such multiresistant strains often exhibit elevated antibiotic resistance levels. (For a discussion of penicillin-binding proteins and the uptake of drugs

Table 2. The amino acid changes and their locations within the protein sequence (69) identified in the development of extended-spectrum β-lactamases. Revised from (8) and reproduced with the author's permission.

β-lactamase	Amino acid at position							
	39	104	164	205	237	238	240	265
TEM-1*	Gln	Glu	Arg	Gln	Ala	Gly	Glu	Thr
TEM-2*	Lys							
TEM-13*	Lys							Met
TEM-3	Lys	Lys				Ser		
TEM-4		Lys				Ser		Met
TEM-5			Ser		Thr		Lys	
TEM-6		Lys	His					
TEM-7	Lys		Ser					
TEM-8	Lys	Lys	Ser			Ser		
TEM-9		Lys	Ser					Met
TEM-10			Ser				Lys	
TEM-11	Lys		His			?		
TEM-12			Ser					
TEM-14	Lys	Lys				Ser		Met
TEM-15		Lys				Ser		
TEM-16	Lys	Lys	His					
TEM-17		Lys						
TEM-18	Lys	Lys						
TEM-19						Ser		
TEM-24	Lys	Lys	Ser		Thr		Lys	
TEM-26		Lys	Ser					
SHV-1*	Gln	Asp	Arg	Arg	Ala	Gly	Glu	Leu
SHV-2						Ser		
SHV-3				Leu		Ser		
SHV-4				Leu		Ser	Lys	
SHV-5						Ser	Lys	

*These are parental types, lacking extended-spectrum activity.

by bacteria, see the accompanying reviews in this issue by H. Nikaido and B. G. Spratt.)

Aminoglycosides. Just as with the penicillin and cephalosporin antibiotics, the introduction and therapeutic use of a series of naturally occurring and semisynthetic aminoglycosides over a 20-year period (1968 to 1988) led to the appearance of multiresistant strains resulting from selection and dissemination of a variety of aminoglycoside resistance determinants. Whereas there is (formally) only one type of chemical modification of β-lactams (hydrolysis of the four-membered ring) that leads to bacterial resistance, a dozen different types of modification are known to be responsible for resistance to the aminoglycosides. When one considers that each of these enzymes has a number of isozymic forms, there must be at least 30 different genes implicated in bacterial resistance to this class of antibiotics. The genes encoding modification of aminoglycosides do not appear to undergo facile mutational changes that generate enzymes with altered substrate activity; in vitro mutagenesis studies have failed to generate extended-spectrum resistance to this class of antibiotics. Rather, the response of bacteria to the introduction of a new aminoglycoside antibiotic is to acquire a different resistance gene. For example, when the use of kanamycin was superseded by a new but structurally related aminoglycoside (gentamicin), a previously unknown class of antibiotic-inactivating enzyme was detected in the gentamicin-resistant strains that appeared in hospitals.

The phylogenic relationships between different aminoglycoside-modifying enzymes is the subject of a review by Shaw and her co-workers, who have collated the nucleotide and protein sequences of the known aminoglycoside acetyltransferases, phosphotransferases, and adenylyltransferases responsible for resistance in both pathogenic bacteria and antibiotic-producing strains (*14*). Different proteins in the same functional class may show as little as 44% amino acid similarity. Rarely do a few point mutations in the aminoglycoside resistance gene suffice to generate a modified enzyme with altered substrate range that would lead to a significant change in antibiotic resistance spectrum (*15*), and so far, such changes have not been identified in clinical isolates, in contrast to the situation with the β-lactamases.

Many Gram-positive pathogens possess an unusual bifunctional aminoglycoside-modifying enzyme, the only reported instance of fused resistance genes. The enzyme encodes acetyl- and phosphotransferase activities based on protein domains acquired from two independent resistance genes (*16*). The hybrid gene is widely distributed among hospital isolates of staphylococci and enterococci and can be assumed to have evolved as a fortuitous gene fusion during the process of insertion of the two resistance genes into the "cloning" site of a transposon.

The therapeutic use of aminoglycoside antibiotics has decreased in recent years, largely because of the introduction of the less toxic broad-spectrum β-lactams. As a result, there has been a relatively limited effort to seek specific inhibitors of the aminoglycoside-modifying enzymes, which might have offered therapeutic potential to extend the effective range of this class of antibiotics. Although some active inhibitors have been identified, none of them has been deemed fit for introduction into clinical practice.

Chloramphenicol acetyltransferases (CATs). CAT genes are widely distributed among bacterial pathogens of all genera. This group of enzymes has been analyzed in great detail by Shaw and his collaborators (*17*); at least a dozen breeds of CAT genes encoding similar but not identical acetyltransferases have been identified (*18*). As with the aminoglycoside-modifying enzymes, the CAT genes are (presumably) of independent derivation, because they could not be linked by a small number of point mutations to a single ancestral gene. Potential origins for the CAT family have yet to be clearly identified. Chloramphenicol use has declined rapidly in many countries (because of toxicity of the drug, which causes depression of bone marrow function leading to blood disorders such as aplastic anemia), and there are now very few indications for which chloramphenicol is the drug of choice. Thus, though chloramphenicol resistance determinants are widespread, their presence is not of major consequence from a therapeutic standpoint in industrialized nations. However, chloramphenicol is an effective broad-spectrum antibiotic and is inexpensive to produce, so it is employed extensively in the Third World for the treatment of a variety of Gram-negative pathogens (*Salmonella, Vibrio*, and *Rickettsia*), and liberal over-the-counter availability of the antibiotic ensures strong selection pressure for the maintenance of chloramphenicol resistance. The type I CAT (encoded by transposon Tn9) has two activities: in addition to catalyzing the acylation of chloramphenicol, the protein forms a tight stochiometric complex with the steroidal antibiotic fusidic acid (*19*); thus sequestered, the latter antibiotic is ineffective. This is the only plasmid-determined resistance to fusidic acid that has been characterized. Notwithstanding their different structures, the two antibiotics bind competitively to the enzyme; interestingly, both chloramphenicol and fusidic acid exert their antibiotic action through inhibition of bacterial protein synthesis. Hence, the type I CAT is bifunctional, determining resistance to two structurally related antibiotics by distinct mechanisms. Given our understanding of the basic biochemistry of the drug and knowledge of the mechanism of chloramphenicol resistance (*20*), it is surprising that more effort has not been put into rational design of chloramphenicol analogs of lower toxicity that would be active against resistant strains. Fluorinated analogs of chloramphenicol with reduced substrate activity for CAT have been produced but have not been afforded extensive clinical study (*21*). The antibiotic has an excellent inhibitory spectrum: Might it be possible to use fusidic acid analogs to inhibit chloramphenicol acylation?

Modification of other antibiotics. A widely used mechanism for the detoxification of cell poisons in eukaryotes is the formation of glutathione adducts. For example, this mechanism is commonly used for herbicide detoxification in plants (*22*); however, in spite of the fact that many microbes generate large quantities of this important thiol, only one example of an antibiotic resistance mechanism of this type has so far been identified in bacteria—that of fosfomycin, a product of a streptomycete that is used clinically in several countries in Europe. Fosfomycin interferes with bacterial cell wall synthesis and is employed in the treatment of sepsis, both alone and in combination with other antimicrobial agents. Transmissible resistance is due to a plasmid-encoded glutathione-S-transferase that catalyzes the formation of an inactive fosfomycin-glutathione adduct (*23*). Two independent genes for fosfomycin resistance have been cloned and sequenced, one from *Serratia marcescens* (*24*) and the other from *Staphylococcus aureus* (*25*); the two genes are unrelated at the sequence level.

There are a number of other examples of transferable resistance mechanisms that involve the covalent modification of antibiotics. For example, O-phosphorylation of erythromycin has been identified in a number of bacterial isolates (*26*), and hydrolytic cleavage of the lactone ring of this class of antibiotics has also been described. The lincosamides (lincomycin and clindamycin) have been shown to be inactivated by enzymic O-nucleotidylation in Gram-positive bacteria. For the macrolides and lincosamides and the related streptogramins (the MLS group) (*27*), such forms of antibiotic inactivation seem (at least for the moment) to be a relatively minor mechanism of resistance. In clinical isolates, enzymic modification of rRNA, rendering the target (ribosome) of the antibiotic refractory to inhibition, is the most prevalent mechanism of resistance to macrolides and,

worldwide, compromises the use of this class of antibiotics in the treatment of Gram-positive infections (*28*).

Origins of Genes Encoding Antibiotic Resistance

Covalent modification of their inhibitory biochemical products is very common in antibiotic-producing bacteria (Table 3). It was the discovery of antibiotic modification as a means of self-protection in the streptomycetes that led to the proposal that antibiotic-producing microbes were the origins of the antibiotic resistance determinants found in other bacteria (*29*). Support for this hypothesis has been provided by nucleic acid and protein sequence comparisons of aminoglycoside resistance determinants from producing organisms and clinical isolate sources (*17, 30*). To date, similar molecular comparisons with other types of resistance determinants have been limited. It should be made clear, however, that producing organisms are not the only potential source of antibiotic resistance mechanisms. As has been pointed out by Udou *et al.* (*31*), Shaw *et al.* (*32*), and Rather *et al.* (*33*), "housekeeping" genes such as the sugar kinases and acyltransferases may have evolved to modify aminoglycoside antibiotics. In addition, a cryptic chromosomal gene for an aminoglycoside acetyltransferase appears to be present in many enterobacteria, as identified by Southern (DNA) hybridization studies (*14*). Piepersberg *et al.* (*34*) and subsequently Martin *et al.* (*35*) had earlier suggested that the genes for protein kinases (and perhaps protein acetyltransferases) were the ancestral sources of some classes of aminoglycoside-modifying enzyme genes.

The first tangible evidence of resistance gene transfer involving antibiotic-producing streptomycetes in a clinical setting has come from Pang *et al.* (*36*). These researchers analyzed human infections of nontuberculous mycobacteria and *Streptomyces* spp.; the infections did not respond to treatment with tetracyclines. Both microbial species contained resistance genes (*tet*K and *tet*L) known to be the basis of tetracycline resistance in Gram-positive bacteria. These resistance determinants promote efflux of the drug and are typically transposon-associated. Surprisingly, the mycobacteria and the streptomycetes both had the tetracycline resistance genes (*otr*A and *otr*B) previously identified in the tetracycline-producing strain, *Streptomyces rimosus* (*30, 37*). Reciprocally, the streptomycetes had acquired the *tet*K and *tet*L genes. Because the latter are clearly "foreign" genes (*tet*K and *tet*L have a G+C content different from those of streptomycete and mycobacterial chromosomal DNA), they must have been acquired as the result of a recent gene transfer. Although this evidence is consistent with resistance gene transfer between the streptomycetes and other bacteria, it is not known which is donor and which is recipient, nor whether the newly acquired tetracycline resistance genes are plasmid or chromosomally encoded. We know that mycobacteria and streptomycetes (both soil microbes) participate in interspecific gene exchange with other bacterial genera by conjugation (*38*). This may exemplify the first step in the dissemination of antibiotic resistance genes to other bacterial species in nature. The acquisition of exogenous antibiotic resistance genes by pathogenic mycobacteria is of considerable concern, because clinically significant antibiotic resistance in mycobacteria is usually associated with mutation; extrachromosomal elements have not previously been implicated (*8, 39*).

It should be apparent from the foregoing discussion of antibiotic modification that there must be a substantial pool of antibiotic resistance genes (or close relatives of these genes) in nature. Gene flux between bacterial replicons and their hosts is likely to be the rule rather than the exception and appears to respond quickly to environmental changes (*40, 41*). This gene pool is readily accessible to bacteria when they are exposed to the strong selective pressure of antibiotic usage—in hospitals, for veterinary and agricultural purposes, and as growth promotants in animal and poultry husbandry. It is a life-or-death situation for microbes—and they have survived. A better knowledge of the components of this gene pool, particularly with respect to what might happen on the introduction of a new chemical entity as an antimicrobial agent, might, on the one hand, permit early warning and subsequent chemical modification of antibiotics to permit them to elude potential resistance mechanisms and, on the other, lead to more prudent use of antibiotics under circumstances where the presence of specific resistance determinants can be predicted. At the present time it is not possible to conceive of ways of avoiding the selection of antibiotic-resistant bacterial mutants that appear during the course of antimicrobial therapy. However, the resistance gene pool would be of no use unless bacteria had the means to access this collection to their advantage. There have been many studies on the mechanisms by which genes can be "picked up" and transferred by interspecific traffic processes.

Evolution of R Plasmids

Studies by Hughes and Datta of plasmids that they isolated from Murray's collection of bacteria preserved from the pre-antibiotic era (*42*) suggest that the appearance of resistance genes is a recent event—that is, the multiresistance plasmids found in pathogens must have been created in the past five decades. What really takes place when a new antimicrobial agent is introduced and plasmid-determined resistance develops within a few years? The most significant component of the process of antibiotic resistance flux in the microbial population is gene pick-up, which has now been emulated in the laboratory.

Largely due to the studies of Hall and

Table 3. Resistance to some clinically useful antibiotics in their producing organisms. In a number of cases, strains have multiple resistance mechanisms for the antibiotic formed (*70*). *Str.*, *Streptomyces*.

Producing strain	Antibiotic	Resistance gene	Mechanism
Str. capreolus	Capreomycin	*cph, cac*	Phosphotransferase, acetyltransferase
Nocardia lactamdurans	Cephamycin	—	β-lactamase (?)*†
Str. venezuelae	Chloramphenicol	—	Hydrolase‡
Str. erythreus	Erythromycin	*erm*	Target modification (ribosome)
Str. fradiae	Fosfomycin	*fos*	Glutathione adduct (?)†
Micromonospora purpurea	Gentamicin	*grm*	Target modification (ribosome)
Str. kanamyceticus	Kanamycin	*aac*	*N*-Acetyltransferase
Str. antibioticus	Oleandomycin	*mgt*	Glycosyltransferase
Str. griseus	Streptomycin	*aph*	*O*-Phosphotransferase
Str. rimosus	Tetracycline	*otr*A, otrB	Efflux, resistant translation system
Str. tenebrarius	Tobramycin	*aac, kgm, kam*	Acetyltransferase, target modification (ribosome)
Str. vinaceus	Viomycin	*vph*	Phosphotransferase

*It is noteworthy that the large majority of streptomycetes produce β-lactamases, although they do not (apparently) make β-lactams. †Evidence that these are true self-protection mechanisms in the producing organisms is not yet available. ‡Although the organism producing chloramphenicol does not make chloramphenicol acetyltransferase, a number of non–chloramphenicol-producing streptomycetes are known to make the enzyme.

Stokes (*43, 44*), we have a good idea of the way in which transposable elements carrying multiple antibiotic resistance genes might be formed. From their studies of the organization of transposable elements, these researchers have identified a key structural constituent of one class of transposons that they named an "integron." The integron is a mobile DNA element with a specific structure consisting of two conserved segments flanking a central region in which "cassettes" that encode functions such as antibiotic resistance can be inserted. The 5′ segment encodes a site-specific recombinase (integrase) and a strong promoter or promoters that ensure expression of the integrated cassettes. The integrase is responsible for the insertion of antibiotic resistance gene cassettes downstream of the promoter; ribosome binding sites are conveniently provided. More than one promoter sequence exists on the element (*45*), and transcription initiation is very efficient and functions in both Gram-negative and Gram-positive bacteria. The 3′ segment carries a gene for sulfonamide resistance (*sul*) and two open reading frames of unknown function. Probably the ancestral integron encoded no antibiotic resistance (Fig. 1).

The ubiquitous presence of *sul* in an element of this type might be surprising, although sulfonamides (a synthetic antimicrobial) have been employed since the mid-1930s and (apart from mercury salts) are the longest used agents for the treatment of infectious diseases. The *sul* gene encodes a dihydropteroate synthase that is refractory to sulfonamide inhibition; the origin of this resistance gene is at present unknown. The resistance gene cassettes are integrated into a specific insertion site in the integron. Typically, in the case of Tn*21*-related transposons, each antibiotic resistance cassette is associated with one of a functional family of closely related, palindromic 59–base pair (bp) elements (or recombination hotspots) located to the 3′ side of the resistance gene (Fig. 1). Integrase-catalyzed insertion of resistance gene cassettes into resident integrons has been demonstrated (*44, 46*). In addition, site-specific deletion and rearrangement of the inserted resistance gene cassettes can result from integrase-catalyzed events (*47*).

Francia and co-workers (*48*) have expanded our understanding of the role of integrons in gene mobilization by showing that the Tn*21* integrase can act on secondary target sites at significant frequencies and so permit the fusion of two R plasmids by interaction between the recombination hotspot of one plasmid and a secondary integrase target site on a second plasmid. The secondary sites are characterized by the degenerate pentanucleotide sequence Ga/tTNa/t. Though the details of the mechanism by which new integrons are then generated from the fusion structure are not established, the use of secondary integration sites could explain how new genes may be inserted into integrons without the necessity for a 59-bp element, as the authors point out.

Analyses of the integron-type transposons provide a good model for the way in which antibiotic resistance genes from various (unknown) sources may be incorporated into an integron by recombination events into mobile elements and hence into bacterial replicons, providing the R plasmids that we know today (Fig. 2). (*49*). However, in bacterial pathogens a variety of transposable elements have been found that undergo different processes of recombinational excision and insertion. It is not known what evolutionary mechanisms are implicated or whether some form of integron-related structure is present in all cases. For the type of integron found in the Tn*21* family, we have plausible models, supported by in vivo and in vitro studies, to provide a modus operandi by which antibiotic resistance genes were (and are) molecularly cloned in the evolution of R plasmids. It would be interesting to examine the collection studied by Hughes and Datta (*42*) to see if they contain integron sequences. A large number of transposable elements carrying virtually all possible combinations of antibiotic resistance genes have been identified (*50*), and nucleotide sequence analysis of multiresistant integrons shows that the inserted resistance gene cassettes differ markedly in codon usage, indicating that the antibiotic resistance determinants are of diverse origins. Microbes are masters at genetic engineering, and heterologous expression vectors of broad host range in the form of integrons were present in bacteria long before they became the vogue for biotechnology companies in the 1980s.

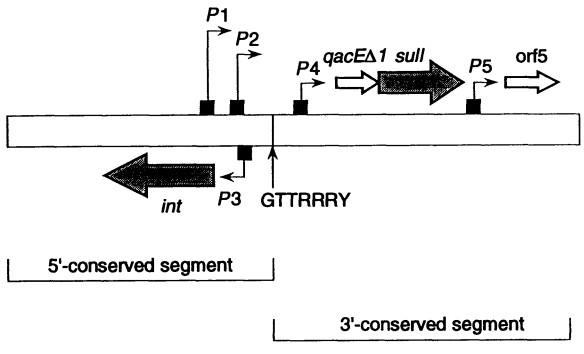

Fig. 1. The general structure of an integron that consists of a 5′ segment that encodes an integrase (*int*) (related to other site-specific recombinases) under the control of promoter *P*3 and a 3′ segment that encodes genes for resistance to quaternary ammonium compounds (*qacEΔ1*) and sulfonamides (*sulI*). The function of the remaining reading frame (orf5) is not known, but the putative product shows some similarity with a puromycin acetyltransferase (*49*). The promoters *P*1 and *P*2 direct the transcription of antibiotic resistance gene cassettes that are integrated between the two conserved segments at the GTTRRRY insertion site of the integron. A large variety of resistance genes may be inserted in different combinations, each with a downstream 59-bp element (*49, 68*), to generate a series of multiresistant transposons and plasmids. It is conceivable that the "ancestral" integron possessed only an integrase gene and an accompanying integration site. Modified figure by Roy and co-workers (*45*), based on the proposal of Stokes and Hall (*43*), published with permission of the author and Elsevier Science Publishers. Abbreviations for the amino acid residues are G, Gly; T, Thr; R, Arg; and Y, Tyr.

Gene Transfer

Given the variety of insertion elements in the generation of R plasmids and the flux of antibiotic resistance genes, what about the intermicrobial traffic of these genetic elements? Horizontal and vertical transfer of antibiotic resistance genes (and genes for metal resistance and bacterial virulence, for example) is now believed to be commonplace in the microbial kingdom (*40*). The prevalent mechanisms of genetic exchange between bacteria are (i) conjugation, (ii) transduction, and (iii) transformation. In addition, variants on these processes have been developed for genetic engineering purposes, such as electrotransformation, electroduction, or protoplast fusion. It is only in recent years that we have begun to better perceive the extent and range of gene transfer in nature. That conjugation occurred between genera as different as the aerobes and anaerobes has been appreciated since 1984, but dogma held that there was a barrier to transfer between Gram-negative and Gram-positive bacteria. However, Trieu-Cuot *et al.* (*51*) dispelled this notion by constructing appropriate multifunctional conjugative vectors that crossed this imagined barrier in either direction. This finding has been confirmed in numerous other studies (*52*). In principle it requires only the addition of a short DNA sequence (*ori*T) to a replicative element to render it mobilizable by conjugation by cis-action of the transfer functions of another plasmid. Subsequently, many different bacterial species have been shown to participate in sex factor–directed mating (even with yeasts and, in one case, plants), which provides

confirmation of the efficacy of interspecific gene transfer by this mechanism (*53, 54*). This was a surprising series of findings, because conjugation was thought to be the most limited process of gene transfer, requiring highly specific interactions between donor and recipient. However, the promiscuous nature of conjugative transfer appears to be the norm, and even bacteria in a moribund state can participate and donate genetic material (*55*).

Though certain steps of the conjugation process vary depending on the bacterial genera (such as diffusible pheromones in *Enterococcus*, different types of pilus and complexity of regulation in Enterobacteriaceae, and broad-host-range transfer in *Staphylococcus*) (*54*), the genetic transfer mechanism is conserved and is based on a family of accessory proteins whose primary function is the movement of macromolecules across cell membranes (*56*). Thus, it is not surprising that conjugation can be viewed as a non–species-specific process.

Transformation of DNA is likely to be an equally significant resistance gene transfer process in nature, as most bacterial genera can be shown to be competent for transformation under some conditions. However, the relative importance of these two mechanisms in environmental transfer cannot be estimated, largely because we are incapable of defining any environment in microbial terms.

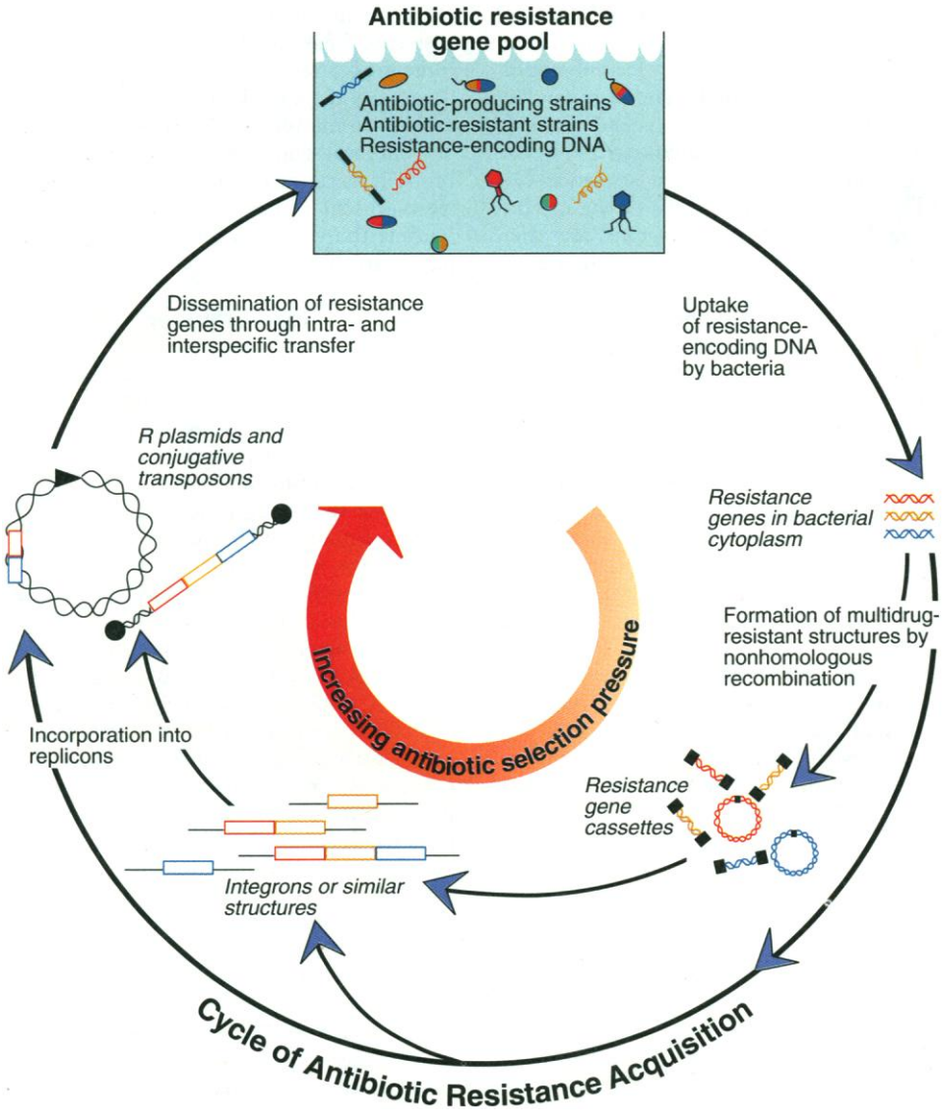

Fig. 2. A scheme showing the route by which antibiotic resistance genes are acquired by bacteria in response to the selection pressure of antibiotic use. The resistance gene pool represents all potential sources of DNA encoding antibiotic resistance determinants in the environment; this includes hospitals, farms, or other microenvironments where antibiotics are used to control bacterial development. After uptake of single- or double-stranded DNA by the bacterial host, the incorporation of the resistance genes into stable replicons (DNA elements capable of autonomous replication) may take place by several different pathways which have not yet been identified. The involvement of integrons, as shown here, has been demonstrated for a large class of transposable elements in the Enterobacteriacae. The resulting resistance plasmids could exist in linear or circular form in bacterial hosts. The final step in the cycle—dissemination—is brought about by one or more of the gene transfer mechanisms discussed in the text.

Laboratory studies of gene transfer have provided the only leads to what goes on in the environment. The multispecies gene exchange that takes place in soil would seem to be a good case for the application of chaos theory to microbiology. Although gene transfer between artificially introduced donor or recipient bacteria with soil microbes can be demonstrated (*57*), we know nothing of the gene traffic in natural environments such as soil and the gastrointestinal tract, which comprise mixtures of a large number of bacterial genera and species. It is likely that the process of gene exchange involves multiple steps—a microbial cascade of transfer, if you will. However, given our current notions of resistance plasmid evolution, if free DNA is involved in the process, transformation is likely to be the first step in the process of gene acquisition in nature.

The conjugative transposons represent a fortuitous combination of two processes important in resistance gene flux in nature. This type of transposon was first identified by Clewell and his co-workers (*58*) in their studies of R plasmids in Gram-positive *Enterococcus faecalis*; since their discovery, this type of dual-function transposon has been found in a variety of Gram-positive bacteria and in the Gram-negative *Bacteroides* (*59*), one of the predominant microbial components of human colonic microflora. In these bacterial species, originally antibiotic-susceptible, clinically significant resistance to a variety of antibiotics has occurred by the acquisition of one or more conjugative transposons. Resistance is subsequently disseminated by cell-to-cell contact among a variety of pathogens that were heretofore treatable with available antibiotics. The enterococcal conjugative transposons have an extremely broad host range of transfer, and transposition of antibiotic resistance genes takes place in many hosts (*60*); the mechanism of transposition has been studied extensively (*61*).

As with a number of other conjugative elements, the conjugative transposons are able to promote the transfer of co-resident R plasmids by a process known as mobilization. A striking characteristic of the *Bacteroides* conjugative transposons (all of which encode tetracycline resistance) is that the antibiotic tetracycline regulates conjugative transfer, mobilization, and transposition (*62*). At least 100-fold increases in gene transfer were observed if bacteria harboring

the transposon were exposed to low concentrations of tetracycline. Similar effects of antibiotics have been observed for conjugative transposons in other bacterial genera (*63*). The implications of these findings are alarming—not only is the expression of the antibiotic resistance gene dependent on the presence of the antibiotic, but the antibiotics provoke the transfer of their own resistance genes! The extent and biochemical nature of this phenomenon is not well understood. A number of different antibiotics have been shown to promote plasmid transfer between different bacteria, and it might even be considered that some antibiotics are bacterial pheromones (*53*). Could this be the primary role of so-called antibiotics in nature? Tetracycline-induced transposition and conjugation in the *Bacteroides* transposons appears to be a specific effect of the antibiotic at the level of gene transcription; however, alternative mechanisms may be involved in the stimulatory effects of antibiotics on other gene transfer systems. For example, subinhibitory concentrations of antibiotics may stimulate cell-to-cell contact by causing subtle changes in bacterial outer membrane structure.

Concluding Remarks

Though reasonable hypotheses for the origins of a variety of antibiotic resistance determinants and their incorporation into stable replicative and transferable forms can be proposed and supported (in part) by laboratory experiments, no confirmatory demonstration flagrante delicto has been possible (*64*). This is likely to be extremely difficult, if not impossible, because one has to consider the complex problem of microbial diversity. At present, microbiologists can identify less than a few percent of the microbes in nature. Antibiotic resistance genes may be harbored by unknown microbial species, and the passage of a given gene to a known pathogen in response to the selective pressure of antibiotic use is likely to be indirect, involving a cascade of gene transfers between a large number of unknown different microbial species by various mechanisms of gene transfer. A complete characterization of this process would be an experimental nightmare, especially when there are likely to be diversified sources of resistance determinants and a variety of mechanisms for their dissemination to unidentifiable microbial hosts.

The genetic ecology of antibiotic resistance is complex and the problems will be difficult to solve; analysis is usually retrospective, and reliable laboratory models of natural situations are difficult to establish. Recent work has suggested that released DNA is quite stable in the environment and readily traverses membrane barriers (*65*); increased stabilization comes from the attachment of DNA to particulate material. Antibiotic preparations themselves are often contaminated with DNA encoding antibiotic resistance genes, thus increasing the chance of arms-length genetic exchange between antibiotic-producing organisms and the very microbes that they are being used to control (*66*). Such considerations, superimposed on our feeble understanding of the nature, extent, and behaviour of microbial populations in the environment, make it unlikely that antibiotic resistance and its transfer can be effectively controlled by other than good clinical practice, which presupposes reliable identification of the pathogen to be treated; after all, the microbial population of this planet has survived successfully over the past 50 years in the face of a relentless onslaught of antimicrobials. The answer to maintaining long-term effective use of therapeutic agents lies in better, more prudent use of antibiotics in human and animal health care, as has been advocated continually since the first discovery of bacteria resistant to antibiotics.

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Prevention of Drug Access to Bacterial Targets: Permeability Barriers and Active Efflux

Hiroshi Nikaido

Some species of bacteria have low-permeability membrane barriers and are thereby "intrinsically" resistant to many antibiotics; they are selected out in the multitude of antibiotics present in the hospital environment and thus cause many hospital-acquired infections. Some strains of originally antibiotic-susceptible species may also acquire resistance through decreases in the permeability of membrane barriers. Another mechanism for preventing access of drugs to targets is the membrane-associated energy-driven efflux, which plays a major role in drug resistance, especially in combination with the permeation barrier. Recent results indicate the existence of bacterial efflux systems of extremely broad substrate specificity, in many ways reminiscent of the multidrug resistance pump of mammalian cells. One such system seems to play a major role in the intrinsic resistance of *Pseudomonas aeruginosa*, a common opportunistic pathogen. As the pharmaceutical industry succeeds in producing agents that can overcome specific mechanisms of bacterial resistance, less specific resistance mechanisms such as permeability barriers and multidrug active efflux may become increasingly significant in the clinical setting.

Antibiotics have been highly effective in the treatment of infectious diseases, and the general population now expects that any bacterial infection will be cured easily by one of these agents. The emergence of resistant bacteria is changing this situation. As described by Neu (*1*), patients in major hospitals staffed by highly competent personnel are dying as a result of infections by resistant bacteria. These resistant bacteria are of two kinds. First, the constant presence of antibiotics in the hospital environment has selected out the unaltered strains of those species that may not possess strong virulence but are intrinsically resistant to a number of antibiotics. These include *Pseudomonas aeruginosa* and *Enterococcus* species, which infect debilitated patients in hospitals as "opportunistic pathogens." Second, there are those bacterial species that are well known for their pathogenicity. Many of these "professional pathogens" used to be exquisitely susceptible to antimicrobial agents. But many years of antibiotic usage have selected out drug-resistant strains, which either contain alterations in their chromosome or have acquired resistance plasmids (R plasmids) or resistance-conferring transposons from another organism.

Bacteria utilize several ingenious mechanisms to develop resistance. These include degradation of the drug, inactivation of the drug by enzymatic modification, and alteration of the drug target (*2*). These mechanisms are all quite specific for a single drug or a single class of drugs. There are, however, more general mechanisms of drug resistance, in which access of the unaltered agent to the target is prevented by the barrier and active transport functions of biological membranes. Thus, an organism can surround itself with a barrier of low permeability in order to decrease the influx of the drug into the cell and can also pump out the drug in an energy-dependent fashion. During the last few decades, the pharmaceutical industry has been successful in producing many synthetic and semisynthetic agents that are able to withstand the action of most of the enzymes that degrade or modify natural antibiotics. Because of this success, the less specific mechanisms such as the permeability barrier and the active efflux are likely to become more important in the clinical setting. Especially noteworthy is the recent observation, presented below, that some bacterial species already possess efflux transporters of very broad substrate specificity, reminiscent of the multidrug resistance (mdr) pump of mammalian cells (*3*).

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Bacterial Species Surrounded by Low-Permeability Barriers

Bacteria are unicellular organisms and their cytoplasm is separated from the external environment by the cytoplasmic membrane. The major permeability barrier in any membrane is the lipid bilayer structure, and its barrier property is inversely correlated with its fluidity (*4*). It is not possible to make the cytoplasmic membrane much less permeable, because this would require decreasing the membrane fluidity and interfering with the proper functioning of membrane proteins. Thus, some bacteria protect themselves by constructing an additional structure that surrounds the cell, outside the cytoplasmic membrane.

Most Gram-positive bacteria are surrounded by a thick peptidoglycan cell wall (Fig. 1). This structure, although mechanically strong, appears to offer little resistance to the diffusion of small molecules such as antibiotics, because its meshwork is too coarse (*5*). In contrast, Gram-negative bacteria, such as *Escherichia coli*, surround themselves with a second membrane, the outer membrane, which functions as an effective barrier (Fig. 1). The outer leaflet of the outer membrane bilayer is composed of an unusual lipid, lipopolysaccharide (LPS), rather than the usual glycerophospholipid found in most other biological membranes. Fatty acid chains present in LPS are all saturated. Because unsaturated fatty acid residues make the interior of the lipid bilayer fluid by preventing the tight