Another look at antibiotic resistance

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Introduction

To many people, antibiotic resistance is associated with failures in antibiotic therapy in hospitals. The object of this short review is to convince the reader that studies of the genetics and biochemistry of antibiotic resistance have revealed many interesting scientific aspects of this problem and a number of useful practical applications; many key questions remain, however! To begin with, genes for resistance to metabolic inhibitors (antibiotics) are quite common among microbes. Although they are most often associated with R-plasmids or transposons in clinical isolates of bacteria or in the production of an antibiotic (in actinomycetes, for example), antibiotic resistance genes are often found in the absence of these relationships and appear to be gratuitous. Nonetheless, the wide distribution of antibiotic resistance phenotypes in prokaryotic and eukaryotic microbes suggests that these genes or their products may play other roles in cell biochemistry. Are antibiotic resistance genes simply to provide the organism with protection mechanisms against the threat of 'natural' antibiotic exposure or do they have other biological functions? If so, what are these functions? Can we even be certain of the biological role of antibiotic resistance genes in antibiotic-producing organisms?

Types of resistance determinants

K. Hotta (personal communication) has suggested that antibiotic resistance can be divided into two types: primary, associated with an antibiotic biosynthetic pathway in a producing micro-organism, and secondary, present in a microbe that does not produce an antibiotic (i.e., the resistance phenotype in this case is apparently not associated with antibiotic synthesis). Antibiotic-

producing streptomycetes often appear to possess both primary and secondary resistance classes, but one must be cautious about this definition, since resistance determinants are often identified by molecular cloning. A so-called primary resistance could be a modification step involved in antibiotic biosynthesis that, when cloned into another cellular background, is expressed as a resistance phenotype. Put in another way, the demonstration of a resistance phenotype by cloning and expressing a gene in a heterologous, sensitive host does not necessarily prove the function of that gene in its native host. A number of modifications occurring as steps of an antibiotic biosynthetic pathway could lead to a resistance phenotype when the genes involved are cloned independently into a naïve background. Such modifications can be early steps in the biosynthetic pathway and may be the equivalent of chemical 'blocking' steps that are removed at the completion of biosynthesis. The roles of secondary resistance functions are not well understood; they may simply be associated with unidentified biosynthetic pathways that are incomplete or not expressed.

Resistance and antibiotic production

In the vast majority of cases, production of a toxic secondary metabolite is associated with an identifiable resistance mechanism in the producing organism. From a practical standpoint the knowledge that resistance determinants are often genetically linked to the cognate biosynthetic genes has permitted the successful molecular cloning of production genes for a number of antibiotics. Although it is generally assumed that the resistance function so identified is a true self-protection mechanism and prevents the organism from committing suicide during antibiotic production, in certain cases the resistance determinant exerts a control function in the biosynthetic pathway, or may be implicated in export of the product.

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Microbial resistance in hospitals

The primary resistance determinants that are clustered with antibiotic production genes in producing organisms must have co-evolved with the biosynthetic genes for secondary metabolites. However, in the case of R-plasmid genes in hospital isolates of bacteria, the secondary determinants are likely to be of more recent origin, probably in the past half-century. They might not have appeared at all, had it not been for the catastrophe which struck the microbial world following man's discovery and widespread use of antibiotics for therapy (and other applications such as animal feeds). Obtaining exact figures is difficult, but it has been estimated that antibiotics have been released into the biosphere to the tune of 10^9 – 10^{10} kg since 1950. As we know to our cost, microbes survived this catastrophe by an unexpected mechanism: inherited resistance mechanisms on R-factors which propagated to protect the microbial hosts.

Biochemistry

Antibiotic resistance in microbes has been studied extensively; the biochemical mechanisms identified to date are listed below.

Modification	Immunity protein
Reduced uptake	Bypass/replacement
Increased efflux	Sequestration
Altered target	

A number of excellent reviews have discussed these mechanisms, their distribution, and their relationship to antibiotic resistance mechanisms in producing organisms and they need not be discussed further here (Jacoby & Archer, 1991). However, recent studies have revealed points of additional interest with respect to some resistance mechanisms. Take, for example, resistance to bleomycin (Gatignol *et al.*, 1987): this compound is a widely used and effective antitumour agent employed under strictly controlled conditions. To all intents and purposes, it has never been employed therapeutically as an antibacterial. Nonetheless, many clinical isolates of bacteria, particularly the staphylococci, are resistant to bleomycin and its derivatives; they carry plasmids that elicit production of a family of small proteins (~14 kDa) which bind specifically and stoichiometrically to the antibiotic. Thus sequestered, the action of bleomycin as a DNA-damaging agent is completely blocked. This mechanism (which bears a strong resemblance to the immunity mechanisms of colicins and ribonucleases, and may be related to these latter as an evolutionary forerunner) has so far been reported for this type of drug only. All of the bleomycin-producing streptomycetes examined produce a similar binding protein, together with a bleomycin acetyltransferase (M. Sugiyama & J.

Davies, unpublished results) which inactivates the drug by modification. The acetyltransferase has not been identified (yet?) in clinical isolates. Both resistance genes are convenient selective markers for prokaryotic and eukaryotic gene transfer studies. It is the rule rather than the exception for resistant strains to encode multiple biochemical resistance mechanisms.

Another antibiotic resistance mechanism worthy of comment is active efflux, which has been characterized for different inhibitors in both producing strains and clinical isolates. Efflux is due to the presence of specialized membrane proteins which fall into two mechanistic classes. One group resembles the multiple drug resistance (*mdr*) determinants found in tumour cell lines resistant to methotrexate and similar anticancer agents (Guilfoile & Hutchinson, 1991). Like the Mdr proteins, the antibiotic resistance efflux proteins are characterized by the presence of several membrane-spanning domains and mediate resistance to more than one type of inhibitor. By contrast, the actual role of *mdr*-like resistance determinants in antibiotic-producing organisms is likely to be the excretion of the drug from the cell. Additional studies of this superfamily of transporters and resistance determinants and their inter-relationships will be of great interest. Given the importance of this type of resistance in cancer, numerous model systems have been established to investigate multiple drug resistance in eukaryotes. For example, the human P glycoprotein has been cloned into yeast. Streptomycete models would appear to be equally valid. In addition to this large family of membrane-associated active export systems, which may well play roles in secretion of antibiotics from their producing organisms, there is another group of resistance determinants that acts to limit the accumulation of antibiotic within the cell cytoplasm. The archetype of this form of antibiotic resistance is the active efflux mechanism found in tetracycline-resistant bacteria: a cytoplasmic membrane protein is responsible for pumping tetracycline rapidly out of the cell. This has been studied extensively by S. B. Levy and his co-workers (Levy, 1992) and is found in resistant clinical isolates and in antibiotic-producing microbes. A similar type of resistance has been recognized for chloramphenicol, methylenomycin and for other antibiotic classes (see Bissonnette *et al.*, 1991). The mechanism does not involve ATP-dependence in the same sense as the Mdr homologues, although energy in the form of a protein concentration gradient is required. Other types of resistance due to active efflux systems employ different energy sources.

Altered targets

In many antibiotic-resistant strains, the drug target in the cell is either modified and fails to bind, or is

unaffected by the antibiotic produced. There are two mechanisms by which an altered target can be produced: by enzymic modification of the target molecule or macromolecule, in which case the entire cellular complement of target is resistant, or by the presence of an additional population of the target refractory to the drug, encoded by an independent ('foreign') gene. In the latter case, the resistant organism possesses a mixed population of a particular enzyme (target), part of which is sensitive and part tolerant to the inhibitor in question. In all such cases the resistant phenotype must be dominant. Numerous examples of this type of resistance have been identified in both producing organisms and clinical isolates. A 'recent' mechanism of this type is the altered peptidoglycan ligase found in vancomycin-resistant clinical isolates (Dutka-Malen *et al.*, 1992).

Resistance to the tetracycline antibiotics is a good example. In addition to the widely disseminated efflux system mentioned above, another resistance mechanism of considerable importance in tetracycline-producing organisms and in Gram-positive and Gram-negative clinical isolates has been characterized. A group of genes [*tet(M)*, (*O*), (*Q*)] of related sequences comprise the determinants of this second class. Studies by M. C. Roberts, V. Burdett, and their co-workers (Roberts & Kenny, 1987; Burdett, 1991) have shown that *tet(M)* encodes a protein very similar in amino acid sequence to bacterial elongation factors; the strongest sequence relationship is with EF-G, and there is a less convincing similarity to EF-Tu also. Recall that tetracycline inhibits protein synthesis by blocking aminoacyl-tRNA binding to the 30S ribosome subunit (A-site), a process that involves EF-Tu; thus the Tet(M) protein might be assumed to be involved in the translation process even though it does not complement EF-Tu or EF-G mutants of *E. coli* and does not appear to have GTPase activity. Nucleotide and protein sequence comparisons of the *tet(O)*, (*M*) and (*Q*) genes and protein elongation factors (Salysers *et al.*, 1990) suggest that this type of tetracycline resistance function has an early evolutionary history. The *tet* resistance determinants must have been inherited from an organism which possesses a resistant elongation factor, or less likely, were derived by mutational alterations of an unknown gene. An unusual aspect of the *tet(M)*-like resistance determinants is that not only are the genes expressed in a wide range of different backgrounds but also that the proteins are functional; this is not usually the case for the protein components of translation (e.g. r-proteins). The tetracycline-producing organism, *Streptomyces rimosus*, has both the tetracycline resistance mechanisms previously mentioned (Doyle *et al.*, 1991).

A better defined class of target alterations responsible for antibiotic resistance are those in which the ribosome (the target for a large number of protein synthesis

inhibitors) becomes insensitive following specific enzymic modification of rRNA. This is a highly significant resistance mechanism in clinical isolates (e.g. erythromycin resistance in staphylococci) and in producing organisms (macrolides, thiostrepton, aminoglycosides).

The existence of this type of resistance mechanism involving a target alteration in producing organisms is worth noticing, since there is no obvious mechanistic relationship to the biosynthetic pathway to the antibiotic. How did the pathway and this type of resistance co-evolve? Perhaps they did not!

Resistance to synthetic antimicrobials

From what I have said up to this point, it is apparent that by studying the details of antibiotic production in microorganisms together with knowledge of mechanisms of action, one ought to be able to make educated guesses to anticipate the possible mechanism(s) of resistance likely to be encountered during the clinical use of antibiotics or their derivatives, before they are so used. Unfortunately, in no case (to my knowledge) have we availed ourselves of this potential, that would permit us to design in advance a series of appropriate blocked antibiotic derivatives. However, it is obvious that such logic does not apply to totally synthetic antimicrobials, unrelated to any (known) natural product. Resistance to the fluoroquinolones is recognized increasingly as a problem in antibacterial therapy, and a number of different mechanisms of resistance have been identified; all appear to arise by mutation during the course of treatment (Wolfson & Hooper, 1989). So far no plasmid-determined fluoroquinolone resistance has been identified, which is not unexpected, since the fluoroquinolones are not natural products – but neither are sulphonamides or trimethoprim! Plasmid-determined resistance to these latter is widespread, and we have no idea as to the origins of the resistance determinants. Nonetheless in the case of the fluoroquinolones, resistance plasmids may be less likely to occur; the dominance/recessive relationships for genes encoding an enzyme required in the processive synthesis of a macromolecule may differ from those for functions involved in simple steps in intermediary metabolism.

Applications of resistance genes

Resistance genes are not all bad! Several useful applications, including those employed in recombinant DNA technology, are listed below.

Selective markers	Secretion systems
Reporter genes	Diagnostic and
Promoters	taxonomic use

Table 1. Antibiotic resistance genes used as selective markers in gene transfer studies

	Prokaryotic	Eukaryotic
Apramycin	+	—
β -Lactams	+	—
Gentamicin	+	—
Streptomycin	+	—
Tetracycline	+	—
Thiostrepton	+	—
Viomycin	+	—
G418	+	+
Hygromycin	+	+
Kanamycin	+	+
Phleomycin/bleomycin	+	+
Puromycin	+	+
Bialaphos	—	+
Cycloheximide	—	+

* Useful for recombinant DNA studies with plants.

In retrospect it is likely that, without the molecular studies of resistance plasmids in the 1960s and 1970s, many aspects of genetic engineering would not have developed so rapidly (Table 1). Studies of resistance mechanisms in producing organisms have provided essential clues on the way some antibiotics work, and also helped to unravel the function of complex macromolecules such as ribosomes. As a case in point, studies of enzymic methylation of ribosomal RNAs in microbes producing translation inhibitors such as the aminoglycosides and macrolides were responsible, to a very large extent, for our current understanding with respect to the role of rRNA in ribosome function (Noller, 1991). This has been confirmed by the demonstration that isolated rRNA is able to catalyse the formation of peptide bonds. Antibiotic resistance phenotypes have been considered valuable in determining taxonomic relationships within microbial genera and species. However, this criterion is not sufficiently accurate in comparison to more specific molecular hybridization methods. The intergeneric mobility of antibiotic resistance genes leads to inadequacies in this diagnostic approach.

Resistance genes and cell function

Other roles of antibiotic resistance determinants in normal cell physiology have been discussed. Bleomycin resistance was mentioned earlier – an odd case from the start, since bleomycin is rarely used as an anti-bacterial, yet many bacterial strains carry resistance to this drug. The best-characterized bleomycin determinants are those encoded by Tn5 (in Gram-negative hosts) and pUB110-related plasmids (in Gram-positive hosts). In

Table 2. Some examples of antibiotic resistance operons

Resistance determinants	Plasmid or transposon
<i>sul aph(3'') (9) aph (6)</i>	pRSF1010
<i>aph(3') bbp aph(6)</i>	Tn5
<i>aac(3) hph</i>	pJR225
<i>aac(6') ant(3'') (9)</i>	pS-a

both cases the drug is sequestered by a bleomycin-binding protein produced in the resistant strain. Recently, Blot *et al.* (1991) have identified another function for the bleomycin resistance determinant of Tn5. It was found that *E. coli* cells carrying the *ble^R* gene (or even a truncated version of the protein) showed increased resistance to the toxic effects of alkylating agents and survived during long periods of incubation after such treatment. How does one rationalize two different phenotypes associated with the same *ble^R* product? The true function of the protein could be a mechanism of cellular survival, the gene being recruited subsequently for bleomycin resistance determination. It has been suggested that antibiotic resistance determinants may play roles in cellular metabolism other than protection of the host. R. J. Wallace and colleagues (Udou *et al.*, 1989) proposed that aminoglycoside acetyltransferase activities detected in mycobacteria may be involved in normal utilization of acetyl coenzyme A. However, this acetyltransferase activity is not inhibited by normal Krebs' cycle intermediates (J. Davies, unpublished results), which is inconsistent with the Wallace proposal.

Resistance gene operons

Continuing with the saga of bleomycin, *ble^R* of Tn5 is transcribed constitutively from the neomycin (APH) promoter as part of an antibiotic resistance gene operon which includes the streptomycin resistance gene. There are a number of examples of such operons (Table 2); one cannot but wonder about the evolutionary pressures leading to co-ordinate regulation for a group of unrelated resistance determinants. Is it due to the need for genetic 'compactness' and efficiency in plasmids or transposons? Operons comprise, most commonly, linked anabolic or catabolic processes co-regulated in an effective and economical manner; it is hard to fathom a case such as Tn5, where three unrelated antibiotic resistance genes (from three different sources?) have been fused into an operon structure. Since antibiotic resistance determinants in clinical isolates are almost certainly post-1940 (Hughes & Datta, 1983), they present a good model for study of operon evolution.

Evolving resistance genes and mutations

One aspect of antibiotic resistance of particular relevance concerns the evolving responses of hospital bacterial populations to new antibiotic selection pressures (e.g. as an existing antibacterial is replaced by a new 'generation' of derivatives). The introduction of structurally modified derivatives of the aminoglycosides, designed to be refractory to inactivation, has led, in all known instances, to the acquisition and dissemination of new resistance genes from sources external to the endogenous hospital population (producing-organisms?). Such patterns have been identified for both Gram-negative and Gram-positive organisms. Simply put, use of a new aminoglycoside selects for the acquisition of an entirely new resistance gene. Biochemical and nucleotide sequence analyses of aminoglycoside genes in clinical isolates have shown the presence of at least eight distinct phosphotransferases, and a similar number of different acetyltransferases. For the latter, the gene families have been classified by the work of R. Hare and G. Miller and their associates (personal communication); the nucleotide sequences of representative genes may differ by as much as 80%. Mutational alteration of the genes to provide modifying enzymes with altered substrate range does not occur *in vivo* or *in vitro*; perhaps the necessary changes are forbidden for reasons of protein structure. A similar situation exists for resistance to macrolides and tetracyclines; in the latter case, a number of *tet*^R genes have been identified, all related by mechanism (antibiotic efflux) but only partially related in sequence (Rubin & Levy, 1990). The 'new' tetracycline resistance determinants could not have arisen from a few simple base changes in a pre-existing resistance gene. New resistance gene acquisition would appear to be the principal route to new antibiotic resistance phenotypes in hospitals – but is it?

Resistance to the β -lactams is clearly an exception. The original hospital β -lactamase determinants were probably acquired from external sources [the actinomycetes, according to the results of nucleotide sequence analyses by R. Kirby (personal communication)]. Nonetheless, during the last 10 years there is evidence of a remarkable natural protein evolution in the case of β -lactamases; more than 30 different mutations have been identified in the original TEM or SHV β -lactamases, providing extended range β -lactamases, which lead to bacterial resistance to newly introduced β -lactam antimicrobials (Jacoby & Medeiros, 1991). In this case 'natural' protein engineering mechanisms are responsible for extending the β -lactamase spectrum to bind or hydrolyse the new generation cephalosporins, which are designed for reduced sensitivity to β -lactamases. In every case, amino acid substitutions changed the substrate

specificity of the β -lactamase proteins. It is of interest that this type of mutational evolution should occur with the genes for β -lactamases and not with other resistance determinants. Can this be explained by differences in effects of point mutations on the substrate specificity of a hydrolytic function compared to a synthetic function? Or is it a reflection of the pharmacokinetic properties of the various classes of drugs or the manner of treatment?

Origins and transfer

The similarity between resistance mechanisms found in different source organisms led to the notion that the secondary resistance determinants on R-plasmids originated from the primary determinants in producing organisms (Table 3). There is increasing circumstantial evidence to support this hypothesis; for example, nucleotide sequence studies of the resistance genes from different sources demonstrate amino acid sequence similarities in the corresponding proteins. In addition, recent studies indicating frequent and promiscuous gene transfer between distantly related bacterial species is consistent with the occurrence of 'illegitimate' exchanges in nature (Mazodier & Davies, 1991). The possibility of direct transfer from a streptomycete to a pseudomonad, for example, may seem unlikely. However, it is not necessary to invoke direct exchanges; it is more reasonable to imagine that distant exchanges between distantly related organisms result from a cascade of transfer between related species. The gene for streptomycin phosphotransferase, which is found in the streptomycin producer *S. griseus*, may be a case in point; a highly similar gene has been identified in *Mycobacterium fortuitum* (J. Coelho de Souza Timm & B. Gicquel, personal communication). This may indicate the beginning of the gene transfer route away from streptomycetes to, ultimately, unrelated Gram-positive bacteria. Related streptomycin phosphotransferase genes are found in *Staphylococcus* spp., and in certain Gram-negative R-plasmids one can imagine interspecific transfers involving many steps in order that the resistance gene arrives in its current host.

Silent resistance genes

As mentioned earlier, antibiotic resistance determinants are sometimes found as 'silent' genes; this applies both in the case of antibiotic-producing streptomycetes and transposon-associated resistance. Expression of the resistance gene may depend on the cytoplasmic (or genetic) background (e.g. the Tn5 streptomycin resistance); in some cases the gene is not expressed because it lacks a functional promoter (the streptothricin resistance

Table 3. Antibiotic resistance determinants in clinical isolates and their relationship to those in streptomycetes

Antibiotic (or class)	Mechanism	Relationship
Aminoglycosides	Modification of drug	Protein sequence similarities (up to 60%)
Bleomycin/phleomycin	Immunity protein	'Domains'
Chloramphenicol	Modification of drug Reduction in uptake	Protein sequence similarities
β -Lactams	Modification of drug	Protein sequence similarities (ca. 40%)
Lincosamides	Modification of drug Modification of target	Mechanism
Macrolides	Modification of drug Modification of target Reduction in uptake	Protein sequence similarities
Phosphomycin	Modification of drug	Protein sequence identity
Streptothricin	Modification of drug	Protein sequence similarities
Tetracycline	Modification of drug Modification of target Reduction in uptake	Protein sequence similarities

gene of Tn7). Some 'silent' resistance genes of streptomycetes are expressed when protoplasts of the host organism are regenerated (Ishikawa *et al.*, 1988); many sets of conditions may permit detection of the resistance phenotype. There may even be natural (environmental) conditions that favour expression of the genes, a specific 'stress', for example.

Movement of resistance genes

While the antibiotic resistance genes in antibiotic-producing organisms are probably the origin of the resistance determinants in clinical isolates, the mechanisms of widespread distribution among hospital pathogens are poorly understood in mechanistic terms. What gene transfer process(es) were used in these transfers? Knowledge of the gene transfer mechanisms that occur in nature, as distinct from the laboratory, is very limited; there have been encouraging developments due to recent interest in risk assessment studies for the release of genetically engineered micro-organisms. Laboratory studies suggest that conjugation is capable of bringing about gene transfer between distantly related microbes. This has been demonstrated with plasmids and conjugative transposons (Gormley & Davies, 1991; Clewell, 1990). Transfer most probably occurs through cascades of microbes and we are unlikely to obtain a better understanding of these events until such time as the nature of the microbes, the behaviour of diverse microbial populations, and the intra- and intercellular movement of antibiotic resistance genes in nature have been better characterized. Studies of resistance gene transfer in hospitals are retrospective; it would be

desirable, one of these days, to examine the process *in flagrante delicto* and track the genes and their hosts as resistance dissemination is actually happening. Unfortunately, studies of the microbiology of diverse populations in natural environments have been unattractive to many funding agencies; it is time to make amends.

I am grateful to the Society for inviting me to present the Fred Griffith Lecture; in addition I wish to thank my many co-workers and colleagues who have contributed to the work and ideas presented. I apologize if I have not mentioned their 'favourite' resistance mechanism.

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