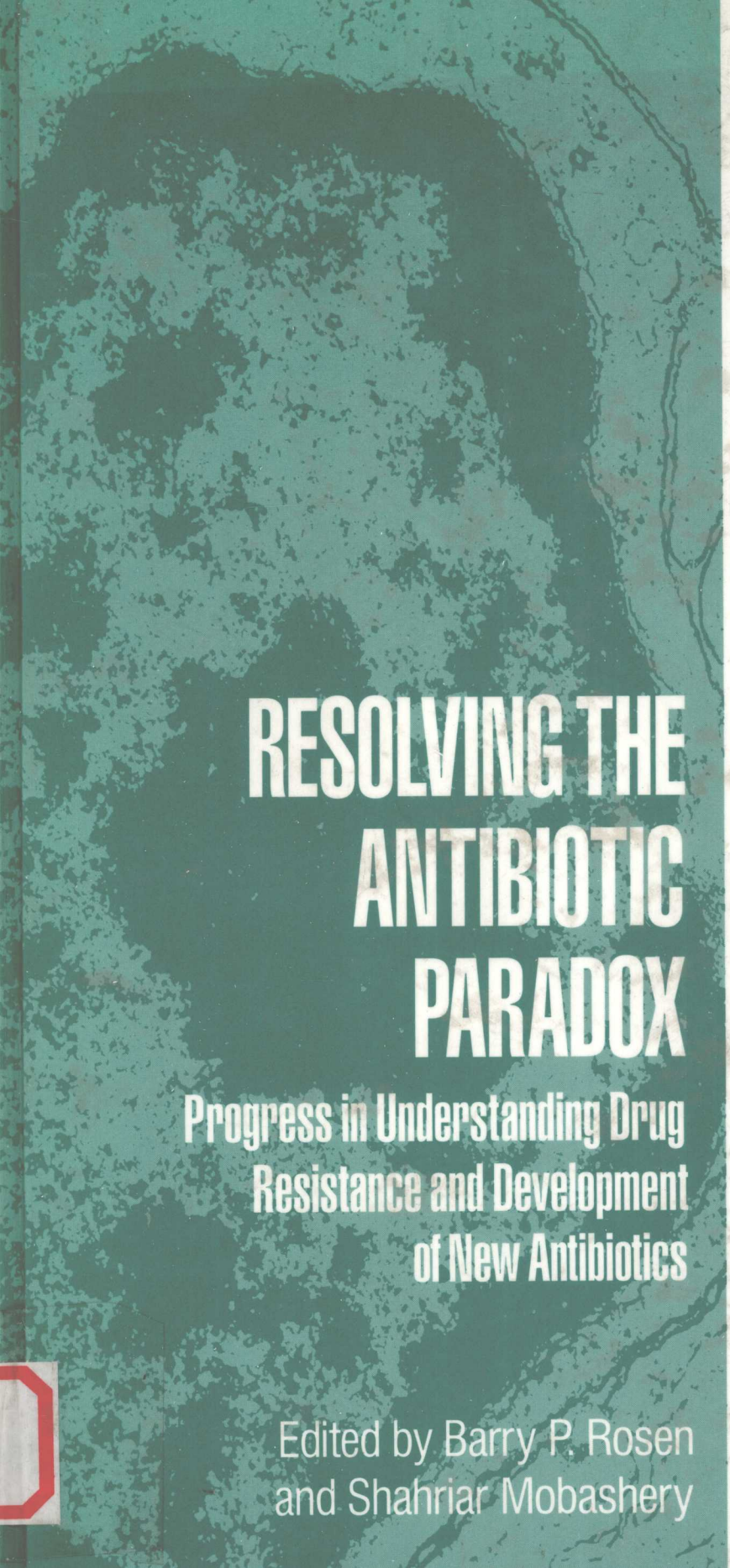

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 456

RESOLVING THE ANTIBIOTIC PARADOX

Progress in Understanding Drug Resistance and Development of New Antibiotics

Edited by Barry P. Rosen and Shahriar Mobashery

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Wayne State University
Detroit, Michigan

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PREFACE

Antibiotic resistance, once a term appreciated only by microbiologists, has become a common topic in the popular press. Stuart Levy, one of the contributors to this collection, bears some of the responsibility for increasing public awareness with the publication of his book "The Antibiotic Paradox" in 1992.* Misuse of antibiotics resulting in increased bacterial resistance had previously been recognized in the infectious disease community. However, Dr. Levy's eloquent public warning about the shrinking efficacy of our antibiotic armamentarium served to alert the lay person to the potential consequences of this demise in useful therapy.

Because of the proliferation of diverse antibiotic classes with increasing potency and broader activity spectra, it had been assumed that any ordinary bacterial infection could be eradicated with the proper selection of drug. However, it has become evident that we are surrounded by resistant bacteria, many of which were introduced unwittingly into our environment through the unnecessary use of antibiotics. When it became evident that a number of people were returning for multiple visits to their family physicians for persistent ear infections or non-responsive bronchitis, questions were raised about the antibiotic treatments that were being prescribed. Bacteria resistant to common antibiotic regimens were being isolated more frequently, often as organisms classified as "multi-resistant" with decreased susceptibilities to two or more structural classes of agents. Nosocomial spread of these resistances was aided by the transmission of plasmid-mediated resistance factors between species. In addition, colonizing bacteria in healthy patients were found to be carrying the same resistance factors in their normal flora. As people are exposed to increasing amounts of antibiotics, either through medical use, or through the overuse of these agents for other purposes, the potential for further mutations becomes greater, resulting in bacteria with even higher resistance. In this book Mazel and Davies present an overview describing the selection of antibiotic resistance, and Lerner presents the resistance picture as seen by a practicing infectious disease specialist. These chapters serve to put the current situation in perspective.

Development of new antibacterial agents has continued for over fifty years, with the early agents based on compounds that were isolated as fermentation products. With the exception of the quinolones, all the classes of antibacterial agents in widespread use today

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* Levy, S. B., 1992, *The Antibiotic Paradox. How Miracle Drugs Are Destroying the Miracle;* Plenum Press, New York.

originated from natural products. Many of these agents such as the β-lactam antibiotics were later tailored to be active against subpopulations of resistant bacteria, based upon specific resistance mechanisms that were deemed to be of medical concern at the time. During the late 1970s and early 1980s an explosion of antibacterial agents targeting the same resistant populations reached the marketplace. As a result, some in the pharmaceutical industry perceived that there was now a glut of available antibiotics, and a number of antibacterial programs were curtailed or diminished, with resources diverted into anti-viral or anti-fungal programs. Those companies that remained actively looking for new drugs were not always searching for novel classes of compounds, so that the impact of many drug discovery programs was low. No obvious break-throughs were identified during this time.

During this perceived hiatus in antibiotic research, many other activities were occurring simultaneously. In the 1980s resistant bacteria were emerging as critical target pathogens, such as vancomycin-resistant enterococci, penicillin-resistant streptococci, methicillin-resistant staphylococci,and Gram-negative bacteria carrying extended-spectrum β-lactamases. Basic research groups in both the academic world and the pharmaceutical industry examined each of these groups of bacteria for novel approaches to therapeutic intervention. In this book both traditional and novel approaches to antimicrobial drug discovery are described to highlight some of the more successful strategies over the past few years.

More conventional drug discovery programs have involved structural modifications of compounds from the traditional classes of natural products. Often a specific type of resistance mechanism has been targeted in an attempt to regain the use of broad-spectrum agents. This kind of approach is discussed in the chapters herein describing novel tetracyclines with activity against tetracycline efflux pumps; the aminoglycoside antibiotics; and the β-lactams that have been designed to circumvent specific β-lactamase inactivation mechanisms. Chimeric drugs such as the quinolonyl lactones have been proposed to capture the activity of both quinolones and cephalosporins. In some cases, well-characterized pathways have been utilized to identify novel targets, as in the case of peptidoglycan biosynthesis.

Some of the more exciting aspects of the current book are the chapters dealing with novel chemical structures shown to have antibacterial activity. Of the agents discussed, the oxazolidinones have progressed the furthest, with linezolid currently in advanced clinical studies. This class of compound with a Gram-positive spectrum of activity represents an advance in structural novelty for antibacterial agents. The diphenolic methanes that have been described as antimicrobial agents working through inhibition of a two component transduction system represent another novel type of agent. Both the oxazolidinones and the bis-phenols are thought to affect bacterial processes that are not associated with the mechanism of action of other antibacterial agents. Therefore, it has been postulated that cross-resistance with other classes of antibiotics should be rare.

Antibacterial activity due to a novel mechanism is the goal of many in the drug discovery community. Resistance issues are perceived to be less, as there would be no drugs in use to pre-select a resistant population. Novel mechanisms that have not been effectively exploited as drug targets include transport mechanisms such as the multi-drug transport and metalloid transport systems described in this book. The newer tetracyclines that inhibit or evade efflux pumps may be included in this group.

Bacterial genomics offers the opportunity for drug discovery scientists to identify novel essential genes that can serve as drug targets. With the proliferation of genomic information that is becoming available, it is only a matter of time and patience before a number of novel targets can be identified. However, risks are always associated with the development of novel drugs. Cross-reactivity between prokaryotic and eukaryotic en-

zymes leading to toxicity is an issue that has already been successfully addressed by the current set of antibacterial agents. But, unless risks are taken, novelty will remain elusive.

In this book the issue of bacterial resistance has been addressed in ways that show both conventional, and precarious, drug discovery approaches. Although one can be assured that any new antibacterial agent will eventually select for a resistant bacterial population, we can hope that this process can be slowed by some of the agents described. Novelty has returned to the antimicrobial discovery process. We continue to hope that at least some of these novel agents will become commercial successes in order to provide us with a new generation of antimicrobial agents.

Karen Bush
R. W. Johnson Pharmaceutical Research Institute
June, 1998

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ANTIBIOTIC RESISTANCE

The Big Picture

Didier Mazel [1] and Julian Davies [2]

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The introduction of antibiotics for the treatment of bacterial diseases in the late 1940s was heralded as one of the great innovations of the modern world (on a par with radio and the internal combustion engine) and led many to believe that infectious diseases would be conquered once and for all. No longer would tuberculosis, dysentery, cholera, pneumonia, enteric diseases inflict their toll on humankind and its social systems -- or so it was thought. The use of antibiotics transformed medical practice to both advantage and disadvantage; previously untreatable diseases were now potentially controllable, but paradoxically, because antibiotics were easy to use and so successful, they may have become a substitute for good clinical practice in some instances. Certainly the use of antibiotics as preventatives (prophy-laxis) in human and animal medicine has had a negative effect on antibiotic therapy overall, the increasing number of treatment failures in hospitals and the community providing abun-dant evidence of this (Ciba, 1997).

It is unlikely that the problem of antibiotic resistance as it exists today could have been anticipated in the early days of antibiotic usage, although there were a few visionaries who recognised the threat and the factors that would promote its appearance.

> It is to be hoped that penicillin will not be abused as were the sulfonamides ... too small doses lead to the production of resistant strains of bacteria. ... There is probably no chemo-therapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring 'fastness' (resistance). Sir Alexander Fleming 1946

> We cannot be sure that the searchers for new drugs and antibiotics will always win the race. However hard and successfully we may work in the search for new drugs we shall therefore continue to labour under discouragement so long as we are faced with the bugbear of drug resistance. The problem is one of microbial biochemistry, physiology and genetics, and can only be solved by work in these fields. Until we understand the problem we shall have no hope of

Resolving the Antibiotic Paradox, edited by Rosen and Mobashery.
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> overcoming it, and until we overcome it we shall have no real sense of security in our chemotherapy. The subject ... is not only of the greatest scientific interest and importance; it has also a background of practical medical urgency. Sir Charles Harington 1957

In more recent years, with widespread resistance to many valuable drugs being reported in learned publications and the popular press, many other physicians and scientists have spoken out against the overuse, imprudent use, and outright abuse of antibiotics (Levy, 1992). There is frequent mention of the end of the antibiotic era or the regression to a pre-antibiotic state in infectious diseases treatment.

In spite of significant increases in knowledge of antibiotic resistance mechanisms and their origins over the past few decades, our understanding is rudimentary, especially with respect to the ecological aspects. Antibiotic resistance is currrently studied by analysis of the end-product -- the organism causing the therapeutic problem. In order to have more control over the development of antibiotic resistance it is essential that an understanding of the earliest stages of the process be obtained. What happens (microbiologically) when a novel antibiotic is introduced for therapeutic use on a large scale? Where and how does resistance first arise and how soon can the consequences be identified? Why is resistance so stable and how do bacteria tolerate their increased genetic load or growth-retarding mutations, in the competition for survival in highly competitive environments? Until these questions can be answered, the approach to antibiotic therapy will remain one of educated guesses with no real hope of controlling infectious disease on a large or even small scale, despite the best-intentioned efforts.

Extensive studies of the antibiotic resistance genes, their expression and the mobile genetic elements that carry them, have led to the genetic and biochemical characterisation of resistance mechanisms in a broad spectrum of bacterial hosts (Davies, 1994). About ten distinct biochemical mechanisms of antibiotic resistance have been identified. The widespread distribution of resistance determinants throughout the microbial population can be readily explained by the pick-up of resistance genes by mobile genetic elements and resulting heterologous gene exchange. The existence of a resistance gene pool that is available to microbes in response to pressures of selection is generally accepted, but the origin of the resistance genes is still unresolved. A number of suggestions have been made that include (1) housekeeping genes (alternate substrates, mutation to new substrate recog-nition, etc.), (2) 'natural' resistance genes in soil communities and (3) antibiotic-producing microbes (self-production). Any or all of these potential sources could be involved, but evidence from comparative studies of the resistance enzymes and their genes supports the notion that antibiotic-producing organisms represent the most likely generalised source (Benveniste and Davies, 1973).

Resistance to the glycopeptide antibiotics (vancomycin, teichoplanin) in the enterococci has increased remarkably in recent years and the resistant strains constitute a significant nosocomial problem. Vancomycin blocks the synthesis of bacterial cell walls by binding to a D-Ala-D-Ala moiety in the growing peptidoglycan structure; resistance arises by the substitution of D-Ala-D-Lac, which is unable to bind the antibiotic effectively, with the result that cell wall synthesis continues in the presence of inhibitor.

The *vanA* gene cluster found in the enterococci contains one of the more complex antibiotic resistance mechanisms so far identified (Leclercq and Courvalin, 1997, Walsh, et al., 1996). It has been demonstrated that the different genes encode functions for (a) altering the vancomycin binding site, (b) removing the endogenous binding sites and (c) regulating the expression of the resistance genes through a two-component system (Figure 1). This gene cluster and the related *vanB* cluster are carried by transposable elements.

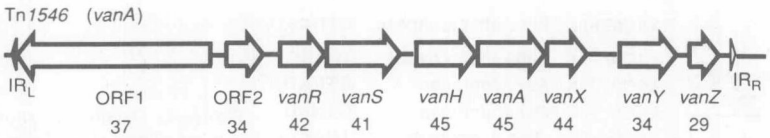

Figure 1. The vancomycin resistance gene cluster of TN*1546*. This gene cluster is responsible for glycopeptide resistance in *Enterococcus faecalis*. The functions of the various genes are: *vanR* and *vanS*, regulator and sensor of two component regulatory systems; *vanH*, dehydrogenase to convert pyruvate to lactate; *vanA*, ligase that inserts lactate into cell wall peptide through ester bond formation; *vanX* and *vanY*, hydrolases to remove the normal vancomycin-binding fragment. Open arrows indicate the open reading frames (ORF1, transposase; ORF2, resolvase). The percentage of guanosine plus cytosine is indicated below each ORF. IR are the inverted repeats. (For more complete information, see Leclercq et al., 1997.)

The evolutionary origin of the *vanA* cluster is of great interest, since it is probable that it was inherited intact rather than being assembled in response to selection. Studies from the laboratory of G. Wright (Marshall, et al., 1997; G. Wright, personal communication), have provided strong evidence that the *vanA*, *H* and *X* genes, whose products are central to the biochemical mechanism of vancomycin resistance, were probably acquired from glycopeptide-producing streptomycetes. The latter organisms possess a group of genes that include a close analogue of the *vanA* gene which, when cloned and expressed in a heterologous host, shows D-Ala-D-Lac synthetic activity. These studies of vancomycin resistance lend credence to the notion that antibiotic-producing bacteria are the foremost source of the antibiotic resistance determinants of bacterial pathogens; this is supported by the finding that antibiotic resistance mechanisms with common biochemical characteristics can be found in both producing organisms and clinical isolates (Table 1).

An interesting exception to the examples presented in this table is the case of sulphonamides and trimethoprim, which are completely synthetic antimicrobials with no natural analogs that inhibit two different steps (dihydropteroate synthase and dihydrofolate reductase) in the biosynthesis of the key metabolic intermediate, folic acid. With these antibiotics bacterial resistance occurs by the acquisition of genes encoding (target) enzymes that are refractory to the two inhibitors. Thus resistant strains possess mechanisms to by-pass both the inhibited steps in the biosynthetic pathway; there is presently no information on the origin of these alien genes.

Table 1. Resistance determinants with biochemical homologues in antibiotic-producing organisms

Antibiotic	Resistance mechanisms*
Penicillins Cephalosporins	ß-lactamases penicillin-binding proteins
Aminoglycosides	acetyltransferases phosphotransferases adenyltransferases
Chloramphenicol	acetyltransferases
Tetracyclines	efflux system ribosomal protection
Macrolides Streptogramins Lincosamines	ribosomal RNA methylation esterases phosphotransferases acetyltransferases
Phosphonates	phosphorylation glutathionylation (?)
Bleomycin	acetyltransferase immunity protein
Vancomycin	D-Ala-D-Lac ligase

*Multiple drug resistance (MDR) efflux systems are common to producing organisms and clinical isolates

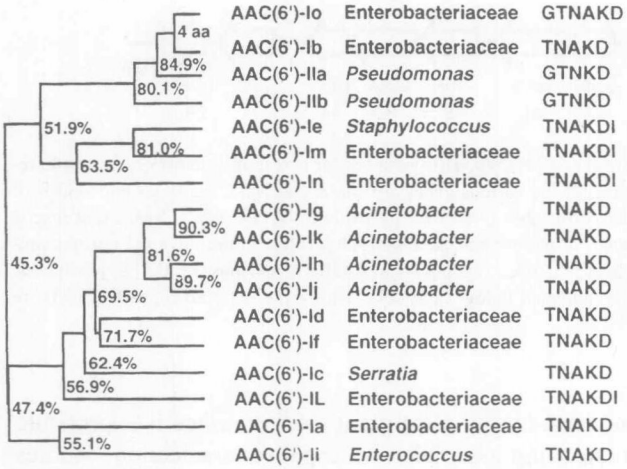

Figure 2. Dendogram showing a comparison of amino acid sequences of the aminoglycoside acetyltransferase (AAC6') family. The numbers represent % similarity in amino acid sequence between different proteins. Also indicated are the source bacterial species and in the right column, the antibiotic resistance spectrum associated with the different enzymes [G: gentamicin; T: tobramycin; N: netilmicin; A: amikacin; K: kanamycin; D: dibekacin; I: isepamicin]. (Figure kindly provided by G.H. Miller.)

Perhaps the best case for the role of heterologous sources as the origin of resistance genes comes from studies of aminoglycoside (AG) resistance. Three types of AG-modifying enzymes have been identified in clinical isolates of bacteria: *N*-acetyltransferases (AAC), *O*-phosphotransferases (APH), and *O*-adenyltransferases (AAD, ANT) (Davies and Wright, 1997). Largely due to the work of the group of Miller, Shaw, and Hare at the Schering-Plough Research Institute, the dynamics of the appearance of different AG enzymes relative to the use of the different AG antibiotics has been tracked world-wide (Miller, et al., 1997). As an example, resistant strains of the 6'-AG acetyltransferases (AAC6') have been analysed and a family of genes that originated in various geographical locations in response to local AG use has been identified. Surprisingly, this AAC6' family (Figure 2) could have been derived only from different (microbial) sources; it is not the result of a series of mutational alterations in a common precursor gene (as is found in the derivation of the extended-spectrum ß-lactamases, coming from one of several ancestors). The same pattern has been demonstrated for several other members of the AG-modifying enzymes (AAC3, APH3', etc.) (Shaw, et al., 1993). It is highly likely that AG-producing organisms contributed a significant number of these genes, since different AG-producing strains are known to elaborate different allelic forms of the same biochemical function. However, it should be pointed out that the producing strains may not have been the sole source of resistance determinants; there is convincing evidence that some of the AG-modifying enzymes are found in other bacterial species and may play a role in cell-wall synthesis (a housekeeping function).

Interestingly, many of the genes for AG-modifying enzymes are found as the components of integrons, and it can be concluded that they were initially inherited as gene cassettes, as is true for several antibiotic resistance genes.

The role of integrons as the major mechanism of acquisition and dissemination of resistance genes in many classes of gram-negative bacteria has been well-established by the work of Hall and Stokes (Recchia and Hall, 1997). Simply put, integrons are naturally-occurring gene expression structures possessing a site-specific integration mechanism for the insertion of open reading frames (gene cassettes) downstream of a strong constitutively-acting promoter (Lévesque, et al., 1995) (Figure 3). Tandem insertions of open reading frames permit the construction of multidrug-resistant elements that can apparently be shuffled to enhance expression of specific determinants.

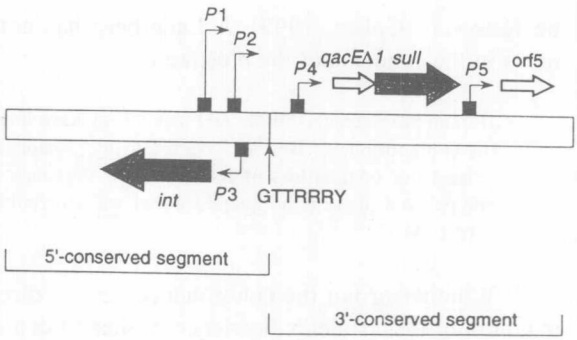

Figure 3. The structure of the elemental integron. The GTTRRRY sequence in the centre is the integration site. The *int* gene is transcribed in a leftward direction from promoter P (lower arrow) and *qac* and *sul* genes rightward from promoter P (upper arrow). Promoters P1 and P2 are responsible for transcription of the antibiotic resistance gene cassettes inserted at the integration site. Produced by permission of P. Roy (see Lévesque et al., 1995).

The integrons have been shown to be essential components of the resistance plasmids associated with the initial epidemic of antibiotic-resistant enterobacterial infections that took place in the early 1950s, which was the first description of the phenomenon of multidrug resistance in bacteria. The actual source of integrons and their associated integrases is unclear; they may well have been derived from temperate bacteriophages, but at present, the three integron integrases that have been identified appear to be quite distinct from the known phage integrases (Capy, et al., 1996). In addition, integrons do not seem to be present in enterobacterial pathogens isolated prior to the introduction of antibiotics for antimicrobial therapy. The "Murray Collection" of pathogenic enterobacteria dating back to 1916 has been screened for the presence of antibiotic-resistance genes and integrons, with negative results (Mazel and Davies, unpublished). Where did the integrons come from?

Although we do not have the answer to this question yet, the finding that the repeated nucleotide sequences typical of pathogenic *Vibrio* sp. (VCRs) are significantly related to integrons would suggest that this type of gene acquisition mechanism pre-dates the introduction of antibiotics. The studies of Mazel and colleagues (Mazel, et al., submitted) have demonstrated that the gene-VCR structure is a substrate for the integron integrases and can be inserted into the *att*I site of an integron. Furthermore, the gene-VCR clusters, which are large structures (super-integrons?) comprising scores of open-reading-frames with (likely) different functions, possess associated integrases which are related phylogenetically to those of the integron. Thus gene-cassette capture systems are common in bacteria, are likely to be ancient (a *V. metschnikovii* isolate from 1888, just 5 years after the isolation of *V. cholerae,* possesses a VCR cluster with accompanying integrase), and must have played roles in the acquisition of genes for pathogenicity and for antibiotic resistance. Might this gene-capture mechanism be responsible also for other acquired functions (such as biodegradation)? The implications of such accumulation mechanisms in the construction of pathogenicity islands (as well as resistance islets) should be readily apparent.

The general conclusion to be drawn is that bacteria have extraordinary genetic flexibility in terms of both mechanisms and functions. Bacterial genomes represent a large natural pool of diverse genetic information that can be accessed under appropriate selection pressures, using a variety of gene acquisition and dissemination mechanisms. Human application of toxic agents on massive scales activates these genetic systems to promote survival of the microbial population. As a result, each and every antibiotic will have a finite lifetime depending on the magnitude and nature of its use; the development of antibiotic resistance is inevitable. Appropriate regulation of antibiotic usage is therefore critical to the continued future of successful antimicrobial therapy, and certain obvious rules must

be followed (Cohen, 1997). J. Lederberg has noted that microbes are formidable opponents in the conquest of the biosphere:

> Human intelligence, culture and technology have left all other plant and animal species out of the competition. ... But we have too many illusions that we can govern ... the microbes that remain our competitors of last resort for domination of the planet. In natural evolutionary competition, there is no guarantee that we will find ourselves the survivor(s). Joshua Lederberg 1994

Whether or not the consequences are *so* dire is a matter of opinion. However, there is no doubt that if medical science wishes to continue its success in the therapy of infectious diseases, a substantial re-evaluation of the use and abuse of antibiotics is needed.

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CLINICAL IMPACT OF ANTIBIOTIC RESISTANCE

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INTRODUCTION

The development of antimicrobial agents for clinical use has brought unquestionable benefits to individuals and to society. Infections that formerly were frequently fatal have become routinely curable. Furthermore, the availability of effective antibiotic therapy has facilitated the employment by physicians of potent immunosuppressive therapy in the control and treatment of other conditions such as cancer and transplant rejection, since resulting infections may be treated and are often suppressed or cured.

Despite these considerable benefits, the utilization of antimicrobial chemotherapy has also produced adverse consequences for individual patients. As with any medication, antibiotics can produce untoward side-effects, such as allergic reactions and impairment of organ function, as of the liver or kidneys. Unlike other medications, however, the use of antibiotics may also have adverse consequences outside the patient. Antibiotic treatment may lead to selection of resistance in the target organisms that cause infection and against which antibiotic therapy is directed. Thus, antibiotics ultimately lose their effectiveness as they are used over time, in contrast to other types of medication.

Occasionally, individual cells of an infecting strain of bacteria may acquire resistance to an antibiotic, either as a result of mutation or by acquisition of foreign genes from a resistant organism. In such a case, in the presence of the antibiotic the resistant cells will proliferate over their susceptible sibling cells, unless they are eliminated by host defenses of the treated patient. When such resistant cells proliferate, they not only exacerbate the patient's infection and complicate his/her treatment, but they may also produce adverse consequences for the wider society. Such resistant populations of pathogens that survive treatment may be shed into the environment, where they may infect other patients and complicate their therapy by limiting the antibiotic options. Over time, such antibiotic-re-

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