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Origins and Evolution of Antibiotic Resistance

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INTRODUCTION

The successful use of any therapeutic agent is compromised by the potential development of tolerance or resistance to that compound from the time it is first employed. This is true for agents used in the treatment of bacterial, fungal, parasitic, and viral infections and for treatment of chronic diseases such as cancer and diabetes; it applies to ailments caused or suffered by any living organisms, including humans, animals, fish, plants, insects, etc. A wide range of biochemical and physiological mechanisms may be responsible for resistance. In the specific case of antimicrobial agents, the complexity of the processes that contribute to emergence and dissemination of resistance cannot be overemphasized, and the lack of basic knowledge on these topics is one of the primary reasons that there has been so little significant achievement in the effective prevention and control of resistance development. Most international, national, and local agencies recognize this serious problem. Many resolutions and recommendations have been propounded, and numerous reports have been written, but to no avail: the development of antibiotic resistance is relentless.

The most striking examples, and probably the most costly in terms of morbidity and mortality, concern bacteria. The discovery of these infectious agents in the late 19th century stimulated the search for appropriate preventative and therapeutic regimens; however, successful treatment came only with the discovery and introduction of antibiotics half a century later. Antibiotics have revolutionized medicine in many respects, and countless lives have been saved; their discovery was a turning point in human history. Regrettably, the use of these wonder drugs has been accompanied by the rapid appearance of resistant strains. Medical pundits are now warning of a return to the preantibiotic era; a recent database lists the existence of more than 20,000 potential resistance genes (r genes) of nearly 400 different types, predicted in the main from available bacterial genome sequences (85). Fortunately, the number existing as functional resistance determinants in pathogens is much smaller.

Many excellent reviews describing the genetics and biochemistry of the origins, evolution, and mechanisms of antibiotic resistance have appeared over the last 60 years. Two of note in recent times are those of Levy and Marshall (82) and White et al. (149). The goal of this short article is not to summarize such a wealth of information but to review the situation as we see it now (most particularly with respect to the origins and evolution of resistance genes) and to provide some personal views on the future of antibiotic therapy of infectious diseases.

Antibiotic discovery, modes of action, and mechanisms of resistance have been productive research topics in academia (27) and, until recently, in the pharmaceutical industry. As natural products, they provide challenging intellectual exercises and surprises with respect to their chemical nature, biosynthetic pathways, evolution, and biochemical mode of action (26, 134). The total synthesis of such natural products in the laboratory is difficult, since these small molecules are often extremely complex in functionality and chirality (98). The antibiotic penicillin was discovered in 1928, but the complete structure of this relatively simple molecule was not revealed until 1949, by the X-ray crystallographic studies of Dorothy

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TABLE 1. Modes of action and resistance mechanisms of commonly used antibiotics[a]

Antibiotic class	Example(s)	Target	Mode(s) of resistance
β-Lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromicin	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C_1 metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C_1 metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target, efflux

[a] Adapted from reference 150a with permission of the publisher.

Crowfoot Hodgkin (73), and was confirmed by total synthesis in 1959 (125). Studies of modes of action have provided biochemical information on ligands and targets throughout antibiotic history (59, 147), and the use of antibiotics as "phenotypic mutants" has been a valuable approach in cell physiology studies (142). The field of chemical biology/genetics grew from studies of those interactions. We have a meager understanding of how antibiotics work, and in only a few instances can the intimate interactions of the small molecule and its macromolecular receptor be interpreted in terms of defined phenotypes. More surprisingly, there is a paucity of knowledge of the natural biological functions of antibiotics, and the evolutionary and ecological aspects of their chemical and biological reactions remain topics of considerable interest and value (3, 8).

To begin, the definition of "antibiotic," as first proposed by Selman Waksman, the discoverer of streptomycin and a pioneer in screening of soils for the presence of biologicals, has been seriously overinterpreted; it is simply a description of a use, a laboratory effect, or an activity of a chemical compound (146). It does not define a class of compound or its natural function, only its application. At the risk of attack from purist colleagues, the generic term "antibiotic" is used here to denote any class of organic molecule that inhibits or kills microbes by specific interactions with bacterial targets, without any consideration of the source of the particular compound or class. Thus, purely synthetic therapeutics are considered antibiotics; after all, they interact with receptors and provoke specific cell responses and biochemical mechanisms of cross-resistance in pathogens. The fluoroquinolones (FQs), sulfonamides, and trimethoprim are good examples.

As in any field of biological study, antibiotic history is replete with misconceptions, misinterpretations, erroneous predictions, and other mistakes that have occasionally led to the truth. This account aspires to focus on the truth. The discovery of antibiotics is rightly considered one of the most significant health-related events of modern times, and not only for its impact on the treatment of infectious diseases. Studies with these compounds have often shown unexpected nonantibiotic effects that indicate a variety of other biological activities; the result has been a significant number of additional therapeutic applications of "antibiotics" as antiviral, antitumor, or anticancer agents. In some cases, the alternative applications have surpassed those of antibiotic activity in importance, such as in the treatment of cardiovascular disease or use as immunosuppressive agents (45).

Unfortunately, the colossal need for these valuable drugs has had a significant environmental downside. In the 60 years since their introduction, millions of metric tons of antibiotics have been produced and employed for a wide variety of purposes. Improvements in production have provided increasingly less expensive compounds that encourage nonprescription and off-label uses. The cost of the oldest and most frequently used antibiotics is (probably) mainly in the packaging. The planet is saturated with these toxic agents, which has of course contributed significantly to the selection of resistant strains. The development of generations of antibiotic-resistant microbes and their distribution in microbial populations throughout the biosphere are the results of many years of unremitting selection pressure from human applications of antibiotics, via underuse, overuse, and misuse. This is not a natural process, but a man-made situation superimposed on nature; there is perhaps no better example of the Darwinian notions of selection and survival.

A LITTLE ANTIBIOTIC HISTORY

Since the introduction in 1937 of the first effective antimicrobials, namely, the sulfonamides, the development of specific mechanisms of resistance has plagued their therapeutic use. Sulfonamide resistance was originally reported in the late 1930s, and the same mechanisms operate some 70 years later. A compilation of the commonly used antibiotics, their modes of action, and resistance mechanisms is shown in Table 1.

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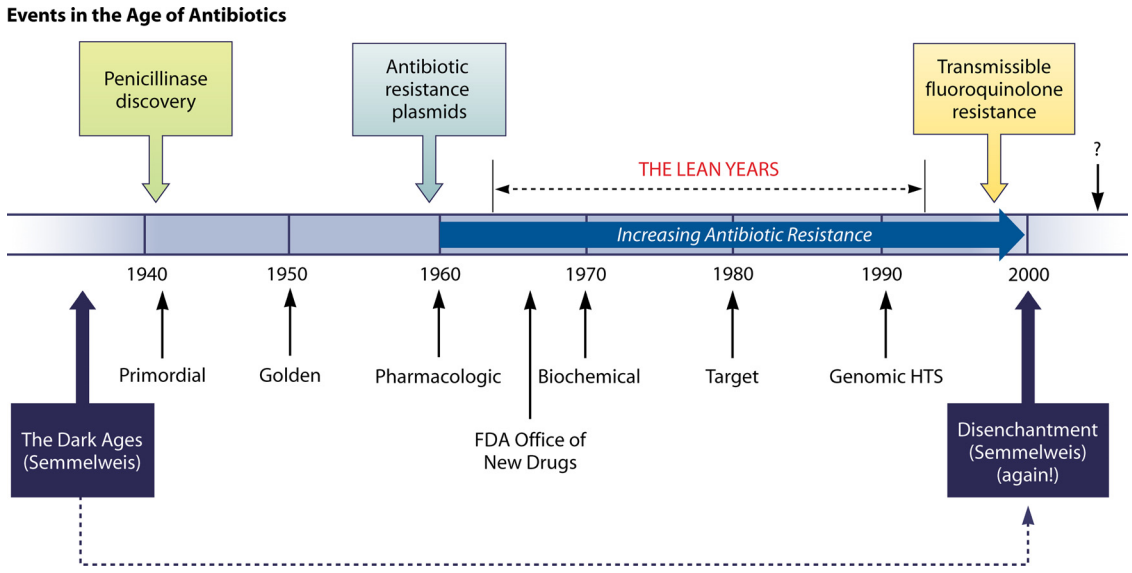


FIG. 1. History of antibiotic discovery and concomitant development of antibiotic resistance. The dark ages, the preantibiotic era; primordial, the advent of chemotherapy, via the sulfonamides; golden, the halcyon years when most of the antibiotics used today were discovered; the lean years, the low point of new antibiotic discovery and development; pharmacologic, attempts were made to understand and improve the use of antibiotics by dosing, administration, etc.; biochemical, knowledge of the biochemical actions of antibiotics and resistance mechanisms led to chemical modification studies to avoid resistance; target, mode-of-action and genetic studies led to efforts to design new compounds; genomic/HTS, genome sequencing methodology was used to predict essential targets for incorporation into high-throughput screening assays; disenchantment, with the failure of the enormous investment in genome-based methods, many companies discontinued their discovery programs. Other milestones in this history include the creation of the FDA Office of New Drugs after the thalidomide disaster led to stricter requirements for drug safety, including the use of antibiotics. This slowed the registration of novel compounds. Before antibiotics were discovered, Semmelweis advocated hand washing as a way of avoiding infection; this practice is now strongly recommended as a method to prevent transmission.

Penicillin was discovered by Alexander Fleming in 1928, and in 1940, several years before the introduction of penicillin as a therapeutic, a bacterial penicillinase was identified by two members of the penicillin discovery team (1). Once the antibiotic was used widely, resistant strains capable of inactivating the drug became prevalent, and synthetic studies were undertaken to modify penicillin chemically to prevent cleavage by penicillinases (β-lactamases). Interestingly, the identification of a bacterial penicillinase before the use of the antibiotic can now be appreciated in the light of recent findings that a large number of antibiotic r genes are components of natural microbial populations (43). Which came first, the antibiotic or resistance?

In the case of streptomycin, introduced in 1944 for the treatment of tuberculosis (TB; "The Great White Plague"), mutant strains of *Mycobacterium tuberculosis* resistant to therapeutic concentrations of the antibiotic were found to arise during patient treatment. As other antibiotics have been discovered and introduced into clinical practice, a similar course of events has ensued. Figure 1 shows the sequence of discovery and resistance development for the major classes of antibiotics. The unexpected identification of genetically transferable antibiotic resistance in Japan in the mid-1950s (initially greeted with skepticism in the West) (39) changed the whole picture by introducing the heretical genetic concept that collections of antibiotic r genes could be disseminated by bacterial conjugation throughout an entire population of bacterial pathogens (with a few notable exceptions) (58, 72).

Only in the past few years has it been appreciated that gene exchange is a universal property of bacteria that has occurred throughout eons of microbial evolution. The discovery of the presence of putative bacterial gene sequences in eukaryotic genomes has heightened awareness of the great importance of horizontal gene transfer (HGT) in genome evolution. Subsequently, other aspects of gene transfer have been revealed by the identification and distribution of genomic islands carrying genes for pathogenicity (69) and other functional gene clusters in different bacterial genera. Not surprisingly, plasmid-mediated transfer of antibiotic resistance has been a major focus of investigation because of its medical and, more recently, practical significance (100).

SUPERBUGS AND SUPERRESISTANCE

Many of the bacterial pathogens associated with epidemics of human disease have evolved into multidrug-resistant (MDR) forms subsequent to antibiotic use. For example, MDR *M. tuberculosis* is a major pathogen found in both developing and industrialized nations and became the 20th-century version of an old pathogen. Other serious infections include nosocomial (hospital-linked) infections with *Acinetobacter baumannii*, *Burkholderia cepacia*, *Campylobacter jejuni*, *Citrobacter freundii*, *Clostridium difficile*, *Enterobacter* spp., *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Serratia* spp., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae*. The term "superbugs" refers to microbes with enhanced morbidity and mortality due to multiple mutations endowing high levels of

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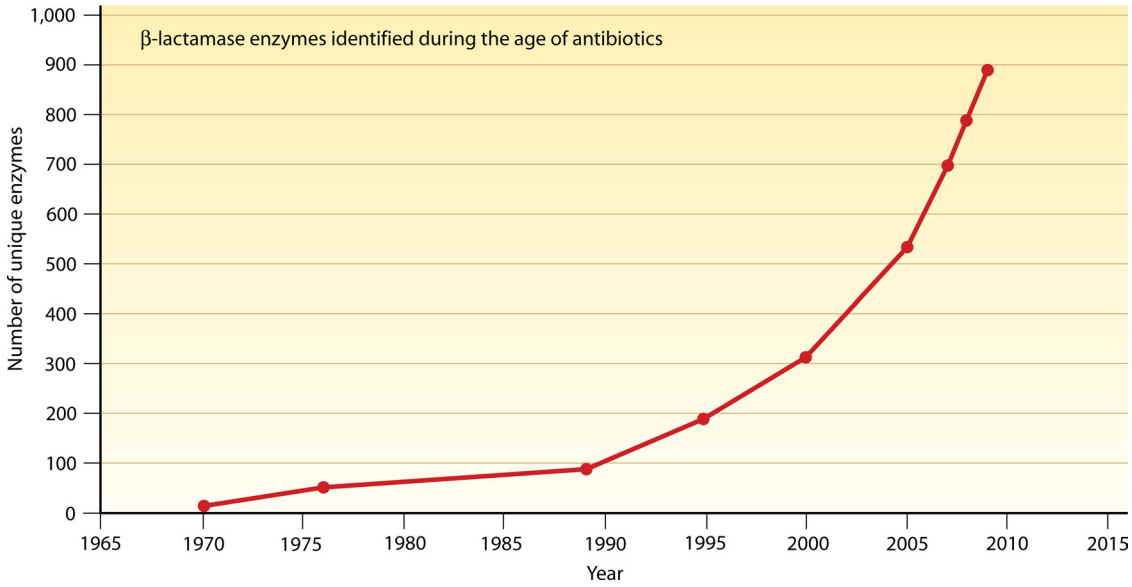


FIG. 2. Numbers of unique β-lactamase enzymes identified since the introduction of the first β-lactam antibiotics. (Up-to-date numbers are courtesy of Karen Bush.)

resistance to the antibiotic classes specifically recommended for their treatment; the therapeutic options for these microbes are reduced, and periods of hospital care are extended and more costly. In some cases, superresistant strains have also acquired increased virulence and enhanced transmissibility. Realistically, antibiotic resistance can be considered a virulence factor.

Tuberculosis is the archetypical human pathogen; it evolved with the human race and currently infects as much as one-third of the world population. While the ground-breaking discoveries of streptomycin and isoniazid provided vital treatments, resistance development was rapid. George Orwell, who suffered from TB while writing the novel *1984*, was apparently infected by an antibiotic-resistant strain of *M. tuberculosis* (122). The introduction of cocktails of anti-TB drugs has become an essential treatment regimen, with considerable success; however, for a variety of reasons, multidrug resistance continues to compromise TB therapy throughout the world. *M. tuberculosis* strains resistant to four or more of the front-line treatments (i.e., extremely drug-resistant [XDR] strains) have appeared and spread rapidly in the last decade or so (124, 130). And now there are TDR strains, which are totally drug resistant (143)! There have been no validated reports of a role for HGT in the development of resistance in *M. tuberculosis*. Antibiotic resistance in *M. tuberculosis* occurs exclusively by spontaneous mutation.

The most prevalent Gram-negative pathogens, such as *Escherichia coli*, *Salmonella enterica*, and *Klebsiella pneumoniae*, cause a variety of diseases in humans and animals, and a strong correlation between antibiotic use in the treatment of these diseases and antibiotic resistance development has been observed over the past half-century. This is especially apparent with the β-lactam class of antibiotics and their related inactivating enzymes, the β-lactamases. At this time, several groups and classes have been identified, comprising up to 1,000 resistance-related β-lactamases (Fig. 2). These include novel classes of genes and their mutant radiations (28, 78, 86, 112, 116). HGT has played a predominant role in the evolution and transmission of resistance to the β-lactam antibiotics among the enteric bacteria in both community and hospital infections.

Concerning hospital-acquired diseases, *Pseudomonas aeruginosa* has evolved from being a burn wound infection into a major nosocomial threat. In this case, again, antibiotic resistance mechanisms evolved coincidentally with the introduction of new antibiotic derivatives, compromising the most effective treatments (such as the β-lactams and aminoglycosides). *P. aeruginosa* is of considerable concern for patients with cystic fibrosis (76); the pathogen is highly persistent and can avoid human immune defenses. Resistance development is associated with the lengthy antibiotic treatment of cystic fibrosis patients.

Acinetobacter baumannii is a more recent Gram-negative pathogen and is also primarily nosocomial. As with the pseudomonads, it comes equipped with a suite of r genes and pathogenicity determinants that results in enhanced rates of mortality and morbidity (107). It is thought that the infectious properties of *Acinetobacter* organisms derive from their robust survival and biodegradation capabilities in the environment; in addition, many strains are naturally competent for DNA uptake and have high rates of natural transformation. *A. baumannii* is evolving rapidly; recent genome sequence studies showed that some derivatives have at least 28 genomic islands encoding antibiotic resistance determinants; more than half of these inserts also encode virulence functions in the form of type IV secretion systems (14, 64).

Currently, the most notorious superbug is the Gram-positive organism *Staphylococcus aureus.* Whether it is the most serious superbug can be debated, since one wonders to what extent its bad reputation is due to its extensive press coverage. *S. aureus* has a close association with humankind: it is carried as a nasal commensal in 30% of the population, and its presence has long been linked to common skin infections such as boils. It does

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not have the historical reputation of *M. tuberculosis*, but in recent years, this multidrug-resistant pathogen has emerged as the major nosocomial infection (50). Following the discovery of penicillin, it seemed that *S. aureus* infections were controllable; however, the respite from resistance was short-lived. The landmark discovery and introduction of methicillin (the first designer antiresistance antibiotic) in 1959 were thought to be a sure defense against the penicillinases, but the appearance of methicillin-resistant *S. aureus* (MRSA) within just 3 years led inexorably to other multiantibiotic-resistant variants, and the acronym now denotes multidrug-resistant *S. aureus*. Relatively recently, MRSA has moved outside the hospital and become a major community-acquired (CA) pathogen, with enhanced virulence and transmission characteristics. CA-MRSA has most of the properties of MRSA, albeit with different *mec* gene clusters, and has acquired new pathogenicity genes, such as the gene encoding the cytotoxic Panton-Valentine leukocidin (44). These are regulated by defined signaling systems (101).

A long-recognized hospital denizen, the toxin-producing anaerobe *Clostridium difficile*, is increasingly found as the cause of severe intestinal infections; recently, hypervirulent toxin-producing strains have been recognized (80, 145). Being a Gram-positive spore former, it is a hardy organism and is readily transmitted by hospital personnel, on equipment, and as aerosols. Its renewed prominence is considered the result of extensive hospital use of antibiotics such as expanded-spectrum cephalosporins, the newer penicillins, and fluoroquinolones that cause significant depletion of the Gram-negative intestinal microflora, thus enhancing *C. difficile* colonization. In other words, these infections are the direct result of antibiotic use.

Superbugs are omnipresent in the biosphere; their consequences are aggravated enormously in volatile situations such as civil unrest, violence, famine, and natural disasters and, of course, by poor or nonexistent hospital practices. Superbugs are not the only microbial threats, but they are recognized as the most menacing with respect to morbidity and mortality worldwide. In terms of the number of infections and consequences, *Vibrio cholerae* should be at the head of the superbug list (84). While fortunately it is not common in industrialized nations, *V. cholerae* is endemic in Asia and South America.

With respect to the global control of endemic and pandemic infectious diseases, a significant problem is the availability of reliable systems for tracking outbreaks of serious infections. Despite the heroic efforts of the World Health Organization, such reporting is nonexistent in many parts of the world. A lack of information concerning the early stages of an epidemic bacterial infection has retarded appropriate remedial action in many cases.

MECHANISMS AND ORIGINS OF ANTIBIOTIC RESISTANCE

The molecular mechanisms of resistance to antibiotics have been studied extensively (Table 1) and have involved investigations of the genetics and biochemistry of many different facets of bacterial cell function (2, 59, 147). In fact, the study of antibiotic action and resistance has contributed significantly to our knowledge of cell structure and function. Resistance processes are widely distributed in the microbial kingdom and have been well described for a variety of commensals (89) and pathogens; most can be disseminated by one or more distinct gene transfer mechanisms. A few of the resistance types that illustrate the difficulties in maintaining effective antibiotic activity in the face of the genetic and biochemical flexibility of bacteria deserve special mention.

Genetic Jugglery

The genes for β-lactamase enzymes are probably the most international in distribution; random mutations of the genes encoding the enzymes have given rise to modified catalysts with increasingly extended spectra of resistance (63). The archetypical plasmid-encoded β-lactamase, TEM, has spawned a huge tribe of related enzyme families, providing ample proof of this adaptability. The β-lactamase genes are ancient (15) and have been found in remote and desolate environments (4), which implies that novel β-lactamases with altered substrate ranges occur in the environment. As another example, a new extended-spectrum β-lactamase (CTX-M) was acquired from environmental *Kluyvera* strains and appeared in the clinic in the 1990s; this was the first enzyme found to hydrolyze expanded-spectrum cephalosporins at a clinically significant level (86). The CTX-M genes and subsequent variants (upwards of 100 different amino acid substitutions have been identified so far) are highly successful at transmission and are a global phenomenon and threat (Fig. 3) (71). Such epidemics of r genes with efficient HGT and rapid mutational radiation are next to impossible to control.

Macrolide antibiotics, such as erythromycin and its successors, were introduced to contend with the problem of methicillin resistance and are widely used for the treatment of Gram-positive infections. Not surprisingly, strains resistant due to a number of different mechanisms are now widely disseminated (120). The macrolides and related antibiotics act by binding at different sites in the peptide exit tunnel of the 50S ribosome subunit. Resistance can occur by modification of the RNA or protein components of the tunnel. A specific rRNA modification that engenders resistance to all antibiotics acting at this site on the ribosome was described recently (88), and this modification is spreading.

Another example of bacterial genetic jugglery comes from the recent appearance of a novel FQ resistance mechanism. When the highly potent FQs were introduced in 1987, a few foolhardy experts predicted that resistance to this new class of gyrase inhibitors was unlikely, since at least two mutations would be required to generate a significant resistance phenotype. It was also suggested that horizontally transmitted FQ resistance was unlikely to occur. However, mutants of the target bacterial gyrase genes and efflux of the FQs from the cell have increasingly been encountered (110). More unexpectedly, a transmissible mechanism of FQ inactivation has made its appearance. This mechanism comes about because one of the many aminoglycoside *N*-acetyltransferases has the capacity to modify a secondary amine on the FQs, leading to reduced activity (46, 99). The latter does not result in high-level FQ resistance but may impart a low-level tolerance that favors the selection of resistance mutations (121). Another unpredicted FQ resistance mechanism is known as Qnr, a widespread family of DNA-binding proteins (105), and is responsible for

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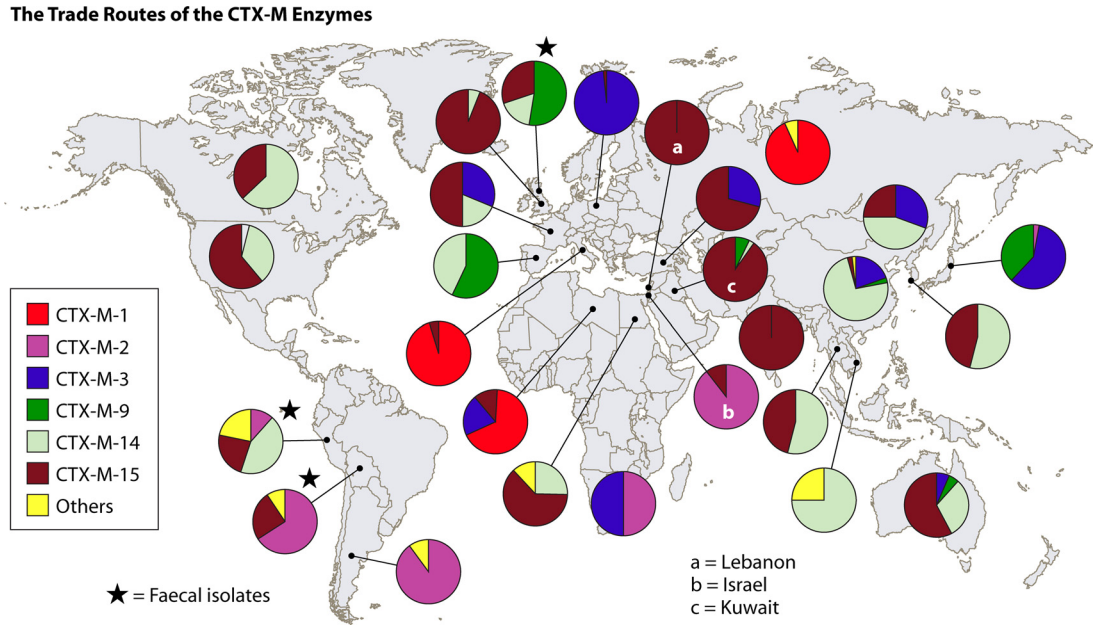


FIG. 3. Worldwide distribution of different classes of CTX-M β-lactamases (first identified in 1989). (Reprinted from reference 71 by permission of Oxford University Press.)

low levels of quinolone resistance (133). We have not heard the end of the quinolone resistance saga. The moral of the story . . . one should not try to second-guess microbes! If resistance is biochemically possible, it will occur.

Intrinsic Resistance

Intrinsic resistance refers to the existence of genes in bacterial genomes that could generate a resistance phenotype, i.e., proto- or quasi-resistance. Different genera, species, strains, etc., exhibit ranges of antibiotic response phenotypes. Since the beginning of this millennium, the availability of genomewide mutagenesis techniques and rapid bacterial genome sequencing has revealed many potential/intrinsic gene functions in bacteria that may lead to resistance phenotypes in clinical situations. For example, a common genetic route to enhanced antibiotic resistance is gene amplification, notably for resistance to the sulfonamides (79) and trimethoprim (25). These studies provide good clues as to what may happen in the future.

Phenotypic analyses of partial or "complete" gene knockout libraries by saturation mutagenesis of bacterial genomes permit the identification of specific mutants eliciting hypersensitivity responses to antibiotics. It is assumed that overexpression of the corresponding wild-type gene would generate a resistance phenotype. Such prognostic studies have been carried out with a number of organisms and have led to the prediction of novel resistance classes. This type of analysis was first done with a partial mutant library of *Acinetobacter baylyi* (64). A more comprehensive survey of the Keio *E. coli* mutant gene library identified a total of 140 distinct isolates that were hypersensitive to a range of different antibiotic classes (137); related studies have been done with *Pseudomonas aeruginosa* (51). Many of the putative "susceptibility" genes identified, such as genes that are genetically recessive, might not lead to a resistance phenotype. Nonetheless, such approaches identify potential r genes and provide information on the systems biology of resistance. RNA microarray analyses of the effects of antibiotics have provided similar predictive information (23). Simply put, increasing the number of copies of the target genes for an antibiotic can lead to reduction in the intracellular concentration of the inhibitor as a result of titration.

Yassin and Mankin used a mutant approach to identify putative target sites for inhibitors of ribosome function (151). Studies with rRNA characterized a number of RNA segments that may be novel targets for small-molecule inhibitors of translation. Such innovative analyses indicate that in spite of suggestions to the contrary, many potential drug targets remain to be exploited in antimicrobial discovery. Predicting resistance reliably—and acting appropriately—would be a valuable approach to extending antibiotic lifetimes (91).

The Resistome

It has been known for some time that bacterial strains resistant to antibiotics can be isolated by plating environmental bacteria on antibiotic-containing media in the laboratory. This is not surprising for antibiotic-producing actinomycetes, since most possess genes encoding resistance to the compounds that they produce. In several cases, the resistance mechanisms have been identified and shown to be specific enzymatic modifications of the antibiotics. Streptomycetes have long been known to produce a variety of β-lactamases that may well be the source of some of the clinical forms of β-lactam resistance (57, 102). As mentioned earlier, environmental *Kluyvera* species have been found to be the origins of the CTX-M genes. In other cases, resistance of producing organisms to their products has been identified as due to efflux systems (68, 111).

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Multiple mechanisms of resistance, as found in the tetracycline producer *Streptomyces rimosus* (109), are frequent in producing bacteria. Based on biochemical and genetic similarities, such resistance mechanisms have presaged those found subsequently in antibiotic-resistant pathogens (18).

In a recent, all-inclusive approach to quantifying the r genes/phenotype density in the environment, Wright and colleagues screened a collection of morphologically distinct spore-forming actinomycetes (including many known antibiotic-producing strains) for resistance to 21 different antibiotics (43). A significant number of strains were resistant to an average of 7 or 8 antibiotics; they were naturally multidrug resistant. The population of r genes in nature is referred to as the environmental antibiotic resistome (17, 150). Clearly, different environments would be expected to vary in the number and type of resistances. Novel resistance mechanisms, as well as many mechanisms related to those found in pathogens, were identified in the collection. This is the best evidence available for the presence of a vast environmental pool of genes with the potential to be captured and expressed as resistance determinants for any overused inhibitor. However, more studies are necessary to establish a strong environment-clinic connection (30).

Similar surveys of other antibiotic-producing bacteria, such as the *Bacillaceae*, pseudomonads, cyanobacteria, and the extensive family of *Actinobacteria* (144), a phylogenetic group known to produce many low-molecular-weight molecules, will be valuable in extending our understanding of the nature of r genes existing in the wild.

The Subsistome

Dantas and coworkers have taken a complementary approach to that of D'Costa et al. by screening soil bacteria for biochemical processes that degrade or inactivate antibiotics (36). Hundreds of strains were randomly isolated from 11 diverse urban and rural soils and tested for the ability to subsist or grow on one or more of 18 different antibiotics as sole carbon and nitrogen sources. Perhaps surprisingly, many strains were isolated that grew efficiently on common antimicrobials, including aminoglycosides, fluoroquinolones, and other classes. Most of the strains identified in this study were proteobacteria, and more than 40% were *Burkholderia* spp.; pseudomonads were also well represented. Obviously, catabolic pathways responsible for antibiotic digestion in nature provide a rich source of potential resistance determinants; additional studies should reveal novel mechanisms of resistance to most antibiotic classes. Work on antibiotic-catabolizing bacteria was reported in the 1970s (53), but the studies of Dantas and colleagues have exposed the full extent and distribution of degradation/r genes in the environment and further verified the roles played by reservoirs of soil bacteria as origins of antibiotic r genes.

Metagenomic Analyses of Environmental Samples

Cloning, PCR, and gene expression techniques have been applied to detect natural r genes in random recombinant clones derived from bacterial DNA libraries from soils and sediments (3, 119). A potential problem is that the identification of functional resistance requires gene expression (transcription and translation) of the cloned genes in a heterologous host; to date, only *E. coli* has been used. Some r genes were identified, but one wonders how many would have been found using a wider range of expression systems and hosts; subsequent global sequencing approaches by D'Costa et al. (43) and Dantas et al. (36) indicate that the number would have been large. Taken together, these studies confirm the existence of many potential antibiotic r genes and mechanisms in nature.

Many questions remain. The roles of these environmental reservoirs in clinical resistance development are still hypothetical, and the primary metabolic functions of proto-/quasi-r genes in microbial populations are as yet unknown. We have little or no evidence that any of the putative r genes identified in these environmental studies have been mobilized into pathogenic bacteria and expressed as resistance phenotypes. If concentrations of antibiotic compounds are essentially undetectable in natural environments, what are the selective pressures for the variety of r genes?

Resistance Due to Anthropogenic Activities

The predominant role of human activities in the generation of environmental reservoirs of antibiotic resistance cannot be disputed. Since the 1940s, ever-increasing amounts of antibiotics designated for human applications have been manufactured, used clinically, released into the environment, and widely disseminated, thus providing constant selection and maintenance pressure for populations of resistant strains in all environments. Obtaining accurate figures on the quantities of antimicrobials produced by the pharmaceutical industry is difficult (it is not in the best interest of pharmaceutical companies to provide this information), but it can be estimated that many millions of metric tons of antibiotic compounds have been released into the biosphere over the last half-century. Since the only available evidence indicates that little in the way of antibiotics is contributed by naturally occurring antibiotic-producing strains in their native environments (65), we must assume that commercial production provides the vast bulk of the antibiotics found in the biosphere. Some alternative uses of antimicrobial agents are as follows: (i) growth promotion/prophylactic use in animals; (ii) therapeutic/prophylactic use in humans; (iii) therapeutic/prophylactic use in aquaculture; (iv) therapeutic/prophylactic use in household pets; (v) pest control/cloning for plants and agriculture; (vi) use as biocides in toiletries and in hand care and household cleaning products; and (vii) culture sterility, cloning, and selection in research and industry. It should be noted that therapeutic use in humans accounts for less than half of all applications of antibiotics produced commercially.

Taking into consideration the large-scale disposal of toxic wastes, metals, disinfectants, biocides, and residues of manufacturing processes, the amounts of noxious xenobiotics released into the biosphere are inestimable. The fact that many of the chemicals disposed are recalcitrant to biodegradation only compounds the issue. The dumping of ciprofloxacin into rivers at levels in excess of 50 kg a day by pharmaceutical manufacturers in Hyderabad, in central India (54), is possibly the most extreme of the horror stories concerning irresponsible disposal; however, similar levels of pollution probably oc-

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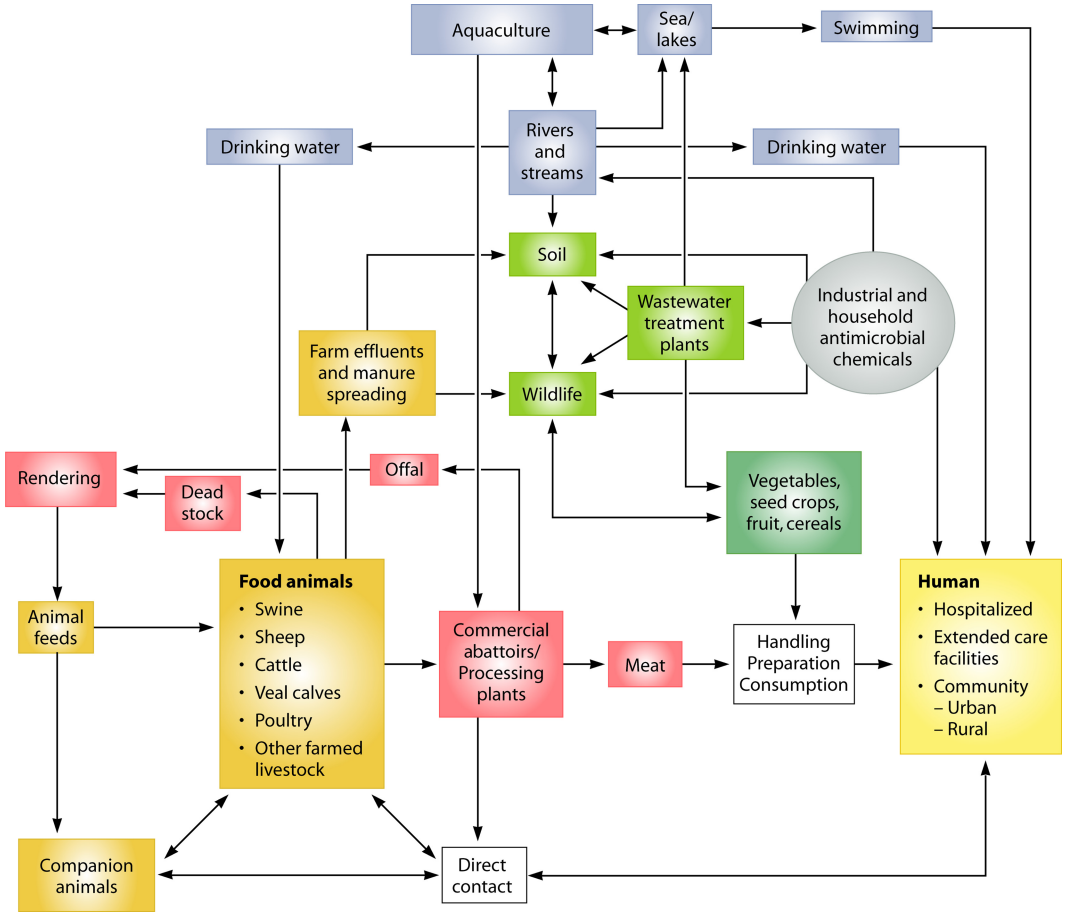


FIG. 4. Dissemination of antibiotics and antibiotic resistance within agriculture, community, hospital, wastewater treatment, and associated environments. (Adapted from reference 49 and reference 83a with permission of the publishers.)

cur (unreported) elsewhere in the world. Quite apart from providing powerful selection for the formation of resistant strains in all bacterial genera (this information has not yet been published), physiological damage to local resident populations of insects, birds, animals, and humans cannot be overestimated (31).

Numerous types of anthropogenic activity, including antibiotic use in agriculture and aquaculture, other nonhuman applications of antibiotics, and waste disposal, create major environmental reserves of resistance (Fig. 4) (49) and, quite probably, of virulence genes and the organisms that harbor them (95). As other examples, genetic and genomic studies of wastewater treatment plants have shown that they are rich reservoirs of r genes and resistant organisms (123, 136); the genes are frequently carried as genomic islands on transmissible plasmids and provide ready sources of resistance determinants. Do these populations have any relationship with resistance in hospitals? Such treatment plants, established for the common good, have become the common bad (13, 34). Steps to ensure better control of antibiotic release and environmental disposal from all users should be immediate and obligatory.

Interesting conundrums have been encountered in investigations of links between antibiotic use and the development of antibiotic resistance. Recent studies have uncovered the presence of antibiotic r genes and even resistance-encoding integrons in the gut flora of peoples who live in isolated areas apparently untouched by modern civilization and not exposed to antibiotic therapies (16, 103, 104). Where did the r genes come from?

GENETICS OF RESISTANCE

The appearance and dissemination of antibiotic-resistant pathogens have stimulated countless studies of the genetic aspects of the different phenomena associated with resistance development, such as gene pickup, heterologous expression, HGT, and mutation (29, 58, 149). The genetics of plasmids is not discussed in any detail here, nor are the interactions between plasmid-encoded and chromosomal resistances, except to say that early preconceptions about the stability, ubiquity, and host ranges of r genes and their vectors have largely become fiction. For example, acquisition of resistance has long been assumed to incur a serious energy cost to the microorganism, and indeed, many resistant mutants may be growth limited under laboratory conditions. As a result, it was considered that multidrug-resistant strains would be unstable and short-lived in the absence of selection (10). However, as frequently demonstrated, laboratory conditions (especially cul-

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ture media) do not duplicate real-life circumstances; available evidence suggests that pathogens with multiple mutations and combinations of r genes evolve and survive successfully *in vivo*. Two recent studies of the development of multimutant, multidrug-resistant *S. aureus* and *M. tuberculosis* provide examples that overturn earlier beliefs. In the first study, isolates from a hospitalized patient treated with vancomycin were sampled at frequent intervals after hospital admission and analyzed by genome sequencing. In the steps to the development of the final (mortal) isolate, 35 mutations could be identified over the course of 3 months (96)! Similarly, it has been reported that genome sequencing of antibiotic-resistant strains of *M. tuberculosis* revealed 29 independent mutations in an MDR strain and 35 mutations in an XDR strain. The functions of these mutations are not understood; they could well be compensatory changes. Such studies emphasize the need for detailed systems biology analyses of resistance development *in situ*.

Resistance Gene Transmission

Essentially any of the accessory genetic elements found in bacteria are capable of acquiring r genes and promoting their transmission; the type of element involved varies with the genus of the pathogen. There are similarities but also clear differences between the Gram-positive and Gram-negative bacteria; nonetheless, plasmid-mediated transmission is far and away the most common mechanism of HGT (100). Surprisingly, bacteriophages carrying antibiotic r genes have rarely been identified in the environment or in hospital isolates of resistant bacteria; however, there is no question about the association of phages with the insertional mechanisms required for the formation of mobile resistance elements and with the functions of chromosomally associated r genes. They are frequently seen as phage "fingerprints" flanking genes encoding resistance or virulence on different vectors. It appears that such events are quite common in *S. aureus* (127).

Gene transmission by conjugation has been studied extensively in the laboratory and in microcosms approximating environmental conditions, and the frequencies of the transfer events often vary significantly. Experiments suggest that frequencies of conjugative transmission in nature are probably several orders of magnitude higher than those under laboratory conditions (129). It has been shown that transfer in the intestinal tracts of animals and humans occurs *ad libitum* (126); it's a bordello down there! Recent studies have demonstrated diverse antibiotic r genes in the human gut microbiome (128).

In the streptococci, meningococci, and related genera, the exchange of both virulence and pathogenicity genes is highly promiscuous; the principal mechanism for DNA traffic appears to be transformation (52, 70, 131). Finally, with respect to direct DNA acquisition in the environment, *Acinetobacter* spp. are naturally competent, and HGT is frequent (14); pathogenic strains typically carry large genomic islands (83, 108). Might *Acinetobacte*r and related environmental genera play roles in the capture and passage of r genes from environment to clinic? Such processes surely involve multiple steps and intermediate bacterial strains, but it has been suggested that heterogeneous gene exchange occurs readily in networks of multihost interactions (48).

Horizontal gene transfer has occurred throughout evolutionary history, and one can consider two independent sets of events, largely differentiated by their time span and the strength of selection pressure. What happened during the evolution of bacteria and other microbes and organisms over several billions of years cannot be compared to the phenomenon of antibiotic resistance development and transfer over the last century. Contemporary selection pressure of antibiotic use and disposal is much more intense; selection is largely for survival in hostile environments rather than for traits providing fitness in slowly evolving populations.

Consistent with the concept of the recent evolution of antibiotic resistance plasmids and multiresistant strains, studies with collections of bacterial pathogens isolated before the "antibiotic era" showed that plasmids were common but r genes were rare (38). Genome sequence analyses of environmental microbes revealed that they are replete with plasmids—mostly large and often carrying multigene pathways responsible for the biodegradation of xenobiotic molecules, such as the polychlorinated phenolic compounds that have been used and distributed widely since the days of the industrial revolution. In summary, what is occurring in our lifetimes is an evolutionary process intensified by anthropogenic influences rather than the slower, random course of natural evolution. The existing processes of gene acquisition, transfer, modification, and expression that were in place are expanding and accelerating in the modern biosphere.

Laboratory studies have characterized numerous genetic mechanisms implicated in the evolution of antibiotic-resistant populations; the roles of plasmids, phages, and transformation are well established, but other processes may exist. For example, bacterial cell-cell fusion might be favored in complex mixed microbial communities, such as those found in biofilms (61). The efficiency of the processes is not critical; selection and the efficiency of heterologous gene expression are likely the most important constraints. However, low-level expression of a potential r gene in a new host may provide partial protection from an antagonist (5); subsequent gene tailoring by mutation with selection would lead to improved expression. Promoter function under environmental conditions is not well understood (32); it appears that promoters of Gram-positive origin can function well in Gram-negative bacteria but that the converse is not often true. Does this imply a favored direction for bacterial gene transfer, as Brisson-Noël et al. have suggested (24)? During therapeutic use, the exposure of bacterial pathogens to high concentrations of antibiotics for extended periods creates severe selection pressure and leads to higher levels of resistance. The pathway from an environmental gene to a clinical r gene is not known, but it obviously occurs with some facility. Knowledge of the intermediate steps in this important process would be revealing—how many steps are there from source to clinic?

In the laboratory, HGT occurs under a variety of conditions and can be enhanced by physical means that facilitate DNA exchange, for example, physical proximity by immobilization on a filter or agar surface, and there are likely numerous other environmental factors that promote gene uptake. It is worth noting that antibiotics, especially at subinhibitory concentrations, may facilitate the process of antibiotic resistance development (41). For example, they have been shown to enhance gene transfer and recombination (35), in part through activat-

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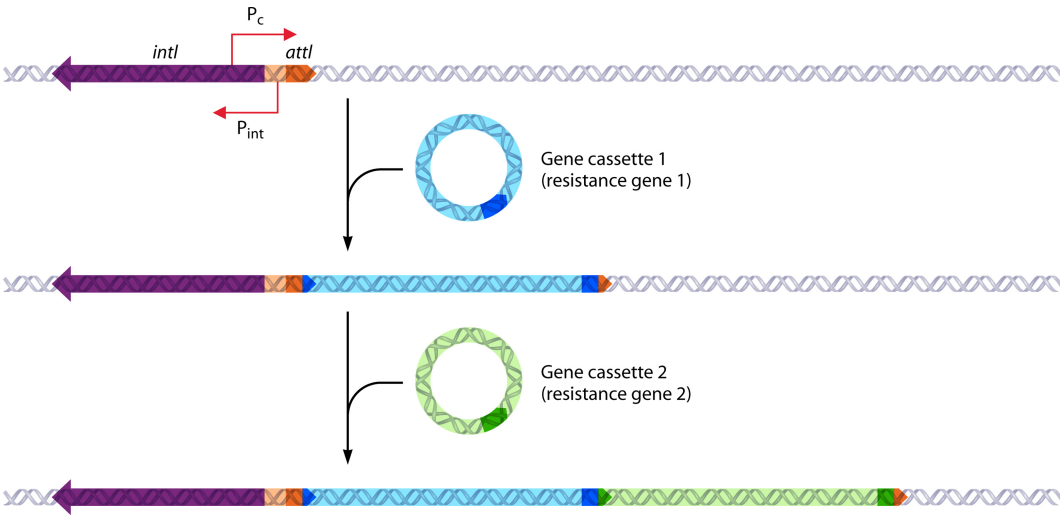


FIG. 5. Integron structure and gene capture mechanism. This figure indicates the basic elements of integrons, as found in bacterial genomes. The structure consists of an integrase (Int) with the P_{int} and P_C promoters in the 3′ end of the gene, with its associated cassette attachment or insertion site (attI). The integrase catalyzes the sequential recombination of circularized gene cassettes into the distal attachment site to create an operon-like arrangement (ant1^r, ant2^r, and so on) of r genes transcribed from the strong P_C promoter (132). Three classes of integrons have been identified that differ in their integrase genes.

ing the SOS system (66, 67); in addition, antimicrobials have been shown to induce phage production from lysogens. Such factors may play important roles in enhancing the frequency of gene exchange in environments such as farms, hospitals, and sewage systems, which provide ideal incubation conditions for r gene acquisition.

On the positive side, it should be noted that studies of antibiotic resistance mechanisms and their associated gene transfer mechanisms in pathogens have played seminal roles in the development of recombinant DNA methods, providing the experimental foundation for the modern biotechnology industry (72). The use of restriction enzymes and plasmid cloning techniques completely transformed biology. The subsequent extension of bacterial recombinant DNA methods to plant, animal, and human genetic manipulations required only minor technical modifications, such as the construction of appropriate bifunctional antibiotics and cognate r genes in pro- and eukaryotes. The applications are truly universal, with increasingly evident benefits to all aspects of pure and applied biology.

Integrons

Integrons are unusual gene acquisition elements that were first identified and characterized by Stokes and Hall in 1987 (132); retrospective analyses have indicated that they were associated with the r genes present in the *Shigella* isolates that characterized the first wave of transferable plasmid-mediated resistance in Japan in the 1950s (83). The "Japanese" plasmids were studied for some 30 years before the integron structure was identified, although resistance determinant components were identified early as composite elements of the plasmids. Figure 5 shows the structure of an integron, its essential functions, and its resistance determinants. Integrons are not themselves mobile genetic elements but can become so in association with a variety of transfer and insertion functions (74). They are critical intermediates in the pickup and expression of r genes (the upstream promoter is highly efficient) and are the source of the majority of the transferable antibiotic r genes found in gammaproteobacteria (60). Recently, it was demonstrated that the process of integron gene capture and expression is activated by the SOS system (37, 66, 67). In a broader view, increasing evidence suggests that components such as integrons and their gene cassettes played important roles in genome evolution and fluidity within the bacterial kingdom (21, 93).

There have been many excellent reviews on the topic of integrons. The complete three-dimensional structure of an integrase has been determined, and the mechanism of r gene cassette acquisition is now well understood (22). Well over 100 cassettes have been identified, covering all the major classes of antibiotics (118). There is functional and genomic evidence that these elements, long thought to be exclusive to Gram-negative bacteria, are present in Gram-positive bacteria as well (97); however, a general role for integrons in antibiotic resistance development in Gram-positive bacteria remains to be established. Most striking is the discovery of very large numbers of integron cassettes in natural environments that do not code for (known) resistance characters. These findings came from high-throughput sequencing of soil metagenomic DNAs and from PCR analyses of DNA samples isolated from diverse soils, sediments, and other natural environments by use of integron-integrase-specific primers (62). Metagenomic analyses of bacterial populations from hospitals, agricultural sites, wastewater treatment plants, and similar environmental sources have revealed many complete integrons with r gene cassettes, underlining the universal importance of integron-mediated gene pickup in resistance evolution. The origins of integrons are not known, although the similarity of sequence between the integrases and bacteriophage recombinases suggests an evolutionary relationship.

Finally, it should be noted that the evolution of different

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types of antibiotic resistance elements in different clinical and natural environments probably involves a variety of integrated genetic processes. Other acquisition and transfer mechanisms have been identified, and the combinatorial nature of the process of resistance development should not be underestimated (46, 139, 148).

ECOLOGICAL ROLES OF ANTIBIOTICS AND ANTIBIOTIC RESISTANCE

Putative antibiotic r genes are omnipresent in natural environments. This raises the question of their natural functions, a topic that has been the subject of several thought-provoking reviews (3, 9, 90). Do they determine antibiotic resistance phenotypes in nature? Are these genes maintained for resistance or for unrelated genetic or biochemical needs? Can we assume that bacteria are constantly exposed to a wide variety of toxins or otherwise inhibitory molecules in the environment? What are the ecological roles of low-molecular-weight natural products identified to have antibiotic activity in the laboratory? They have numerous sources, such as products of the degradation of natural polymers in nutrient conversions, plant products, antibiotic compounds from insects and fungi, and general organic decay. Plants produce many compounds that inhibit bacterial growth in the rhizosphere.

In addition, the environment contains many products that are man-made and/or triggered by human contamination, e.g., petroleum chemicals, solvents, the products and waste of industrial processes, garbage, etc. Since the beginnings of the industrial revolution, humankind has dumped ever-increasing amounts of organic and inorganic toxins into streams, rivers, seas, oceans, land, and air. Heavy metals are frequently present in soils. Arsenic, mercury, and iodine were used industrially and, prior to the discovery of antibiotics, as medicinals; under some circumstances, they are still employed as such. The major bacterial solution to toxic challenges takes the form of multivalent pumping systems that prevent intracellular accumulation of structurally diverse bactericidal and bacteriostatic substances (111, 113). Actinomycetes and other microbes producing antibiotics and bioactive small molecules invariably possess multiple efflux systems (94), as demonstrated for the tetracycline-producing organism *Streptomyces rimosus* (109). The coexistence of production and resistance functions has been confirmed extensively in recent studies of antibiotic biosynthetic gene clusters and examinations of the genome sequences from producing strains (33, 42).

With the exception of nonspecific efflux systems, the potential antibiotic resistance determinants found in antibiotic-producing strains are generally associated with structural types or modes of action. It has been suggested that these resistance mechanisms are for "self-protection" of the host, on the assumption that the producer would self-destruct if it started to make its antibiotic product (75). However, this notion has not been proven.

The production of an antibiotic in the laboratory is routinely assayed by inhibitory activity against bacterial strains of laboratory or clinical origin. Because traditional inhibition assays showed that they are produced late in the growth phase of the microbe, antibiotics have been called "secondary metabolites"; they appear to play no role in normal growth of the host. In addition, the so-called secondary compounds appear to be produced at undetectable concentrations in early exponential phases of growth. Laboratory studies indicate that streptomycetes producing small molecules pass through a transition phase during growth in flask cultures that is associated with the onset of significant developmental changes, including sporulation and antibiotic production. Such interpretations of the relationship of growth-associated processes to small-molecule production may apply only under laboratory and industrial conditions.

The quasi-r genes associated with small-molecule biosynthetic clusters could have other metabolic and regulatory processes. Antibiotic resistance is highly pleiotropic in character. Is it possible that other selective pressures—the expression of efflux or influx systems, for example—might lead to strains resistant to an antibiotic? Pleiotropic interactions can also derive from changes in the distributed metabolic pathways that are networked in cells; an alteration in the concentration of one enzyme or protein could lead to adjustments in processes concerned with microbial community networking (141).

Mutations in ribosomal protein genes leading to antibiotic resistance have a number of extraribosomal effects (mistranslation, temperature sensitivity, phage propagation, etc.) that influence cell function. Different selective pressures may lead to mutations that coincidentally confer a level of antibiotic resistance. An antibiotic resistance phenotype does not necessarily occur solely in response to antibiotic selection.

Whether the microbial products identified to have antibiotic activity do function as antibiotics in natural environments is a moot question. As mentioned earlier, the word "antibiotic" was coined by soil microbiologist Selman Waksman, the Nobel Prize-winning discoverer of streptomycin. He and his distinguished team of researchers (including Mary and Hubert Lechevalier and Boyd Woodruff) isolated hundreds of actinomycetes from different soils and subsequently identified compounds with antibiotic activity in the laboratory (streptothricin, neomycin, actinomycin D, etc.). These discoveries were the genesis of the antibiotic industry. Waksman described an antibiotic as "a compound produced by a microbe that kills or inhibits the growth of another microbe." Subsequently, he must have realized that this was an anthropocentric viewpoint, for he stated that "one is forced to conclude that antibiotics play no role in modifying or influencing the living processes that exist in nature" (146). Unfortunately, the word "antibiotic" had become fixed, defining both compounds and activities. All low-molecular-weight inhibitors from nature were called antibiotics.

Over the last century, studies of microbial natural product function have been predicated on "useful" clinical applications or chemistry. Cellular targets (receptors) have been identified and detailed studies of the biochemistry of inhibitory action carried out (59). However, biological and ecological considerations of the roles of these compounds have been rare. In the case of compounds with antibiotic activity, the principal interest was the MIC, an anthropocentric concept if ever there was one. Some efforts were made to detect antibiotic activity in soil environments (65); however, the fact that the results were negative was possibly a disappointment but was of no concern.

In recent times, studies of microbes in natural environments have provided drastically altered concepts of the natural lifestyles and functions of bacteria (for example, they do not grow

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as isolated, single colonies in the wild), and questions have been asked about the possible roles of the large number of bioactive microbial compounds that are produced. Although the ability to isolate single bacterial colonies on agar was critical to bacterial identification and the study of pathogenicity, this practice has actually delayed the development of microbial ecology. Nowadays, emphasis is being placed on investigations of the interactions within complex bacterial communities (microbiomes) in different environments, as many diseases occur as the result of polymicrobial infections.

Considering again the ubiquity of the quasi-r genes found in nature, if antibiosis is not a common function, what are the roles of these genes? It is well known that antibiotic activity is only one of the biological properties of bioactive small molecules. They exhibit extensive pleiotropy/multifunctionality and most likely are involved in cell-cell signaling within and between bacteria and other organisms in the environment (fungi, plants, insects, and even human and animal hosts). Do the quasi-resistance mechanisms provide the means for attenuating cell-cell interactions, natural degradation pathways, or other functions (41)? Penicillinases have been implicated in cell wall turnover (77, 140). Efflux pumps are promiscuous, and a variety of low-molecular-weight compounds with limited structural similarities may be substrates for the same pump (113).

The Parvome (the World of Small Molecules)

The main conclusion to be derived from the previous discussion is that there is lamentable ignorance of the roles of many millions of low-molecular-weight organic compounds that are produced by bacteria, other microbes, and plants. Their production requires well-defined biochemical pathways and involves biosynthetic gene clusters that are frequently larger than 100 kb. The study of the myriad aspects of small-molecule biology deserves attention. As a collective noun for the infinite world of bioactive small molecules (usually less than 3,000 Da) produced by living organisms, we have coined the word "parvome," a combination of *parv-* (Greek prefix: small) and *-ome* (Latin suffix: group).

To start, answers are needed to key ecological questions. What are the origins of small bioactive organic molecules such as antibiotics? What are the natural roles of these compounds? An even more intriguing question concerns the evolution of the complex biosynthetic pathways of all the bioactive compounds produced in nature. The structural components of antibiotics appear to have existed in the biosphere for billions of years, as evidenced by the number of primordial amino acid derivatives (many of them components of nonribosomal peptides) found in meteorites and by products from "prebiotic" reaction conditions (40). Baltz has calculated that the biosynthetic pathway for a polyketide molecule such as erythromycin may have evolved as many as 800 million years ago (12), and the streptomycin biosynthetic pathway is at least 600 million years old.

HOW TO CONTROL OR REDUCE ANTIBIOTIC RESISTANCE DEVELOPMENT

By any consideration, the most serious consequence of the use of antibiotics is the concomitant development of resistant strains; this has prompted continuous efforts to exert control over antibiotic usage. Erythromycin was an early example; introduced as an alternative to penicillin for the treatment of *S. aureus* in Boston City Hospital in the early 1950s, it was completely withdrawn after less than a year because 70% of all the *S. aureus* isolates were found to have become erythromycin resistant. The same was observed with chlortetracycline and chloramphenicol and, subsequently, with other antibiotics (55).

It is clear that antibiotic resistance seems inevitable. What steps can be taken to prevent or at least delay this process? Over the years, many different solutions have been proposed by knowledgeable experts and all the major international health groups (e.g., WHO and the CDC). Among the proposals for action are strict controls on antibiotic use by humans, requiring accurate prescriptions (no use of antibiotics to treat colds and other viral infections), no delivery of antibiotics without a doctor's prescription (reducing needless use of antibiotics), and controlled therapeutic use in animal husbandry and agriculture. Interestingly, the Swann recommendations of 1969 (135) were the first to call for a ban on nontherapeutic use in animals and agriculture, a reasonable but highly contentious suggestion that has been impossible to enforce in many countries to this day. Deception has played a role in this failure; many of the antimicrobials approved for treatment of humans are given to animals under the cover of different names for different uses, as described in the *Report of the Advisory Committee on Animal Antimicrobial Use Data Collection in the United States of the Alliance for the Prudent Use of Antibiotics* (47). Although the Netherlands and Scandinavia have successfully reduced resistance levels, it is clear that restriction of antibiotic use is difficult to implement on a global scale. Universal adherence to the suggested rules for restraint could have a positive effect, but would resistance be eliminated? Almost certainly not. See the most recent report (of many), *Antibiotic Resistance*: *an Ecological Perspective on an Old Problem* (6). However, if well-considered restrictions and rules for usage were supported by a pipeline of structurally novel antibiotics and semisynthetics designed to be refractory to resistance mechanisms, one could expect some significant and lasting improvements in the treatment of infectious diseases.

Past history provides recurrent warnings. Following its introduction in the United States in the 1950s, penicillin was available over the counter for almost 10 years before prescriptions were required. Thus, we can assume that a "core" population of antibiotic-resistant strains was established by the early 1960s in most industrialized nations. Transmission of plasmid-encoded resistance mechanisms that developed during that period contributed to international dissemination.

The situation today is clearly more complex. In many developing nations, antibiotic use is relatively uncontrolled. Commonly used antimicrobials are comparatively inexpensive in these nations (often costing 10- to 30-fold less than the same drugs in industrialized nations, although they are not necessarily of the same purity or authenticity). In addition, it has been customary for western pharmaceutical companies to distribute antibiotics that are no longer effective or not approved in Europe or North America to developing nations.

On the side of success, mode-of-action-guided chemical modifications of compounds such as aminoglycosides, β-lactams, macrolides, and other antibiotic classes have resulted in active derivatives that are refractory to one or more of the

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known resistance mechanisms. However, the target for resistance function cannot be modified or removed completely without affecting antibiotic activity. Novel semisynthetic compounds generated by such chemical modifications of antibiotic core structures have extended the useful life of several classes, such as methicillin (oxacillin), the macrolide azithromycin, and the modified aminoglycoside amikacin, among others. But this approach does little more than buy time. The r genes evolve in response to new selection pressures, and since multiple mechanisms of resistance exist for every class of antibiotic, the avoidance of each and every modification is impossible. In addition, in some cases, chemical modification of antimicrobials has led to enhanced toxicity.

As mentioned earlier, the ability to pump antibiotics out of cells is a common feature of most environmental microbes and their pathogenic relatives and is the most widespread form of resistance to most classes of antibiotics. Devising compounds that interfere with efflux of active inhibitors from the cell is an attractive strategy for the design of modified or combination therapeutics (87, 111). Unfortunately, in spite of considerable effort, very few effective compounds have been obtained, and only one or two have come close to market. This approach is clearly viable, but for the time being, it remains little more than a pipe dream.

Over the years, there has been much discussion of "cycling" antibiotics to try to reduce selection pressures for resistance and thus prolong the useful life of compounds; this involves the periodic replacement of front-line antibiotics with alternative structural classes in hospitals (19, 92). Cycling does not provide a long-term solution, however, since resistant strains never disappear from the resident population; when related antibiotics are reintroduced, the problem strains (or r genes) are quickly reselected. In large hospital complexes, it may be difficult to decontaminate the "infected" intensive care centers appropriately while cycling between different antibiotics. Has this approach been given a fair test? What might the experience be in more easily controlled situations?

A related tactic involves treatment with combinations of inhibitory compounds that have different modes of action. This combinatorial approach (a fluoroquinolone plus a macrolide or a β-lactam plus an aminoglycoside or tetracycline) has been used in the past to overcome resistance and has also been applied with success in the treatment of diseases such as cancer and HIV infection. However, detailed pharmacodynamic information is essential, and regulatory issues need to be resolved before standardized combinations of antibiotics can be used in routine practice. For example, how does one guarantee that in a mix of two or more active compounds, all arrive at the site of infection at the predetermined concentration range for maximum synergy (and not simply additive effects)? Nonetheless, with seriously life-threatening infections in hospitals, drastic measures must be taken, and a variety of antibiotic combinations are frequently used. Is it possible that older and/or unused (or even discarded) antibiotics might be rehabilitated for "last-resort" use in rational combinations to overcome multidrug-resistant bacterial infections, as some studies have suggested (152)?

Many strategies for avoiding, inhibiting, or bypassing resistance mechanisms in pathogens have been attempted. The most notable successes in such endeavors have been with the β-lactam antibiotics. Clavulanic acid and related compounds are potent inhibitors of β-lactamase enzymes and are frequently used in combination with the β-lactam antibiotics. These combinations have been highly effective (117), but bacteria have found a way to outsmart us: a number of β-lactamases that are refractory to inhibition by clavulanate have appeared (138). To date, research to extend this approach to other classes of antibiotics has not been successful. This poses another interesting ecological question—given that β-lactamases are common in nature, what are the roles of the natural β-lactamase inhibitors, such as clavulanic acid?

It has also been proposed that inhibitors of bacterial virulence could be used to arrest the disease process and thus do away with the requirement for antibiotics. This elegant solution appears to have an advantage over antibiosis in that selection for resistance (survival in the host) might not occur because the growth of the infecting organism would not be impaired. Some success has been obtained in small-animal models, but more extensive studies are essential if this therapy is to be validated (11). Other nonantibiotic approaches for the treatment of bacterial diseases involve stimulation or recruitment of the innate immune system of the host (56). Recent advances in our understanding of the roles of the human gut microbiome in innate immunity may lead to other therapeutic options (115).

This review has ignored (among other things) one of the major aspects of the control of bacterial diseases, i.e., prevention. In an ideal world with effective vaccines against all infectious diseases, the use of antibiotics would be reduced drastically and hopefully limited to surgical procedures in hospitals under strict controls. However, despite years of effort, there are few widely used antibacterial vaccines (7). The success of the pneumococcal vaccine is a model of what can be achieved. Can one hope that more extensive and focused efforts will make it possible to create reliable vaccines effective against *E. coli*, *V. cholerae*, *S. aureus*, *Acinetobacter*, and others?

CONCLUSIONS

The importance and value of antibiotics cannot be overestimated; we are totally dependent on them for the treatment of infectious diseases, and they should never be considered mere commodities. In addition to their use in the treatment of infectious diseases, antibiotics are critical to the success of advanced surgical procedures, including organ and prosthetic transplants.

Notwithstanding all good intentions to control antibiotic usage (but limited action), there is little doubt that the situation with respect to antibiotic resistance is grim. Resistance mechanisms are pandemic and create an enormous clinical and financial burden on health care systems worldwide. There are no simple solutions to the problem. Decisive actions that require significant commitment and enforcement are never popular, even if lives can be saved. Fortunately, not all bacterial pathogens are resistant all of the time, and many respond to empirical treatment with antimicrobial agents administered in the community. Success is perhaps due to luck rather than to good judgment.

Given the many imponderables, the best one can expect is that all physicians and health care centers provide their pa-

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tients with environments that are resistance-free by taking stricter measures in infection control and antibiotic use. This must be backed up by efforts to prevent dumping of antibiotics into the environment through sewer systems; complete destruction of antibiotics before disposal should be common practice.

It is vital that there should be absolutely no letup in the search for new antimicrobial agents (20). Despite the negative attitude of big pharma, the microbial parvome is nowhere near being exhausted in the search for new antimicrobials. Likewise, many uninvestigated drug targets exist in bacterial pathogens. Current knowledge of inhibitor-target and inhibitor-resistance interactions is not at the point where effective new compounds can be designed or screened with confidence; more studies of these processes at the structural level will surely provide new leads. Systems biology approaches are uncovering new types of metabolic interactions and providing nonreductionist explanations for many aspects of antibiotic modes of action and resistance (81, 152). With increasing application of such interactive genome-associated studies, it can be anticipated that new and valid targets will be identified and tested for inhibitor responses by proper consideration of both the direct and indirect functions of genes. The tragedy is that most pharmaceutical companies are now shirking the responsibilities of their own business missions. The onus is on academia to furnish information on the multifunctional aspects of microbial network interactions that will provide the discovery tools of the future.

There is no perfect antibiotic, and once the most appropriate uses of any new compound are identified, it is essential that prescription of the antibiotic be restricted to those uses. This means that defined "niche" antibiotics should be developed as a class separate from broad-spectrum agents. Given the increasing knowledge of environmental reservoirs of resistance, it should now be possible to have early warning of potential resistance mechanisms to new or old antibiotics and thus prepare for problems in the clinic in a proactive manner. It is incumbent on us to renew a concerted offensive that takes full advantage of new understanding and technologies (12, 106, 114). If not, the preantibiotic era awaits our descendants.

ACKNOWLEDGMENTS

We apologize to the authors of many significant papers in the field for not citing their work. There have been upwards of 200,000 references on resistance to antibiotics since the 1950s, and our choice, though selective, was not intended to be exclusive.

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