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Antibiotic Resistance Mechanisms in Bacteria: Biochemical and Genetic Aspects

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Summary

Since the discovery and subsequent widespread use of antibiotics, a variety of bacterial species of human and animal origin have developed numerous mechanisms that render bacteria resistant to some, and in certain cases to nearly all antibiotics. There are many important pathogens that are resistant to multiple antibiotic classes, and infections caused by multidrug resistant (MDR) organisms are limiting treatment options and compromising effective therapy. So the emergence of antibiotic-resistant pathogens in bacterial populations is a relevant field of study in molecular and evolutionary biology, and in medical practice. There are two main aspects to the biology of antimicrobial resistance. One is concerned with the development, acquisition and spread of the resistance gene itself. The other is the specific biochemical mechanism conveyed by this resistance gene. In this review we present some recent data on molecular mechanisms of antibiotic resistance.

Key words: antibiotic resistance, multidrug resistance, antibiotic inactivation, target modification, drug efflux, membrane permeability changes, hypermutators, horizontal gene transfer

Introduction

Infections have been the major cause of disease throughout the history of human population. With the introduction of antibiotics, it was thought that this problem should disappear. However, bacteria have been able to evolve to become resistant to antibiotics (*1–3*). The increase in antibiotic resistance has been attributed to a combination of microbial characteristics, the selective pressure of antibiotic use and social and technical changes that enhance the transmission of resistant organisms. The growing threat from resistant organisms calls for concerted action to prevent the emergence of new resistant strains and the spread of existing ones (*4*).

Recent extensive reviews on the application of antibiotics in human and veterinary medicine (*5–7*), agriculture (*8*) and aquaculture (*9*) have documented the enrichment of antibiotic-resistant bacteria. Many procedures, use and misuse of antibiotics in man have resulted in antibiotic-resistant bacteria. The nutritive and therapeutic antibiotic treatment of farm animals amounts to a half of the world's antibiotic output and has also resulted in antibiotic-resistant bacteria. Evidence is accumulating to support the hypothesis that antibiotic-resistant bacteria from poultry, pigs and cattle enter the food supply, can be found in human food (*10–13*), colonize human digestive tract and transfer resistance genes to human commensals.

There have been very few systematic studies to investigate the acquired antibiotic resistance in lactic acid bacteria (LAB) of food origin. However, they are lately expanding due to increased interest in probiotic lactic acid bacteria and genetic modification of LAB. When LAB live in a biotope regularly challenged by antibiotics (human or animal intestine, bovine udder), the acquired

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antibiotic resistance is found in *Enterococcus*, *Lactococcus* and *Lactobacillus* species (*14–16*). The resistant bacteria may interact with the resident human microflora and possibly transfer or acquire antibiotic resistance determinants by horizontal gene transfer. Large numbers of probiotic bacteria are consumed to maintain and restore the microbial balance in the intestines. It must be kept in mind that they have a potential to transfer antibiotic resistances to pathogenic bacteria. For these and other applications the safety aspects of these bacteria are of concern, including the presence of potentially transferable antibiotic resistances (*14–17*).

Bacteria that normally reside in the human colon can transfer resistance genes among themselves (*18–21*). This type of transfer becomes a huge problem when these harmless commensal bacteria transform into pathogens (*22*). The environment is replete with drug resistance genes, among both pathogen and commensal bacteria. Once acquired, resistance genes are not easily lost. Instead, they become a relatively stable part of a genome. Additional resistance determinants may join those already prevailing, thus broadening the multidrug resistance phenotype and further diminishing treatment options (*23–25*). An increasing number of bacterial isolates is resistant to practically all available therapeutic agents. Multidrug resistance has been demonstrated in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Shigella dysenteriae*, *Enterococcus faecium*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Xanthomonas* and *Burkholderia* (*26*).

Thus, the emergence of antibiotic resistance in bacterial populations is a relevant field of study in molecular and evolutionary biology as well as in medical practice. Here we present recent data on bacterial resistance to antibiotics. We will focus on the molecular mechanisms of antibiotic resistance and genetic parameters involved in development, acquisition and spread of resistance genes.

Modes of Antibiotic Action

Three conditions must be met for an antibiotic to be effective against bacteria: *i)* a susceptible antibiotic target must exist in the cell, *ii)* the antibiotic must reach the target in sufficient quantity, and *iii)* the antibiotic must not be inactivated or modified (*27,28*).

Understanding antibiotic resistance mechanisms requires an understanding of where antibiotics exert their effect. There are five major modes of antibiotic mechanisms of activity and here are some examples.

Interference with cell wall synthesis

β-lactam antibiotics such as penicillins and cephalosporins interfere with enzymes required for the synthesis of the peptidoglycan layer. Glycopeptides (vancomycin, teicoplanin, oritavancin) target the bacterial cell wall by binding to the D-alanyl-D-alanine termini of the peptidoglycan chain, thereby preventing the cross-linking steps. Telavancin, a novel rapidly bactericidal lipoglycopeptide, inhibits peptidoglycan biosynthesis through preferential targeting of transglycosylation (*29,30*).

Inhibition of protein synthesis

Macrolides bind to the 50S ribosomal subunit and interfere with the elongation of nascent polypeptide chains. Aminoglycosides inhibit initiation of protein synthesis and bind to the 30S ribosomal subunit. Chloramphenicol binds to the 50S ribosomal subunit blocking peptidyltransferase reaction. Tetracyclines inhibit protein synthesis by binding to 30S subunit of ribosome, thereby weakening the ribosome-tRNA interaction. The semisynthetic tetracycline derivatives, colloquially termed the glycylglycines, act at the bacterial ribosome to arrest translation. The glycylglycines bind the ribosome more tightly than previous tetracyclines, so that the TetM resistance factor is unable to displace them from this site, hence TetM is unable to protect the ribosomes from the action of these new drugs. The TetA-mediated efflux system is ineffective against the glycylglycines, as they are not substrates for the transporter. The oxazolidinones, one of the newest classes of antibiotics, interact with the A site of the bacterial ribosome where they should interfere with the placement of the aminoacyl-tRNA (*29, 31*).

Interference with nucleic acid synthesis

Rifampicin interferes with a DNA-directed RNA polymerase. Quinolones disrupt DNA synthesis by interference with type II topoisomerases DNA gyrase and topoisomerase IV during replication and by causing double strand breaks (*29*).

Inhibition of a metabolic pathway

The sulfonamides (*e.g.* sulfamethoxazole) and trimethoprim each block the key steps in folate synthesis, which is a cofactor in the biosynthesis of nucleotides, the building blocks of DNA and RNA (*29*).

Disorganizing of the cell membrane

The primary site of action is the cytoplasmic membrane of Gram-positive bacteria, or the inner membrane of Gram-negative bacteria. It is postulated that polymyxins exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial content. The cyclic lipopeptide daptomycin displays rapid bactericidal activity by binding to the cytoplasmic membrane in a calcium-dependent manner and oligomerizing in the membrane, leading to an efflux of potassium from the bacterial cell and cell death (*32,33*).

Biochemistry of Antibiotic Resistance

Understanding the mechanisms of resistance has become a significant biochemical issue over the past several years and nowadays there is a large pool of information about how bacteria can develop drug resistance (*34–36*). Biochemical and genetic aspects of antibiotic resistance mechanisms in bacteria are shown in Fig. 1.

Although the manner of acquisition of resistance may vary among bacterial species, resistance is created by only a few mechanisms: (*i*) Antibiotic inactivation –

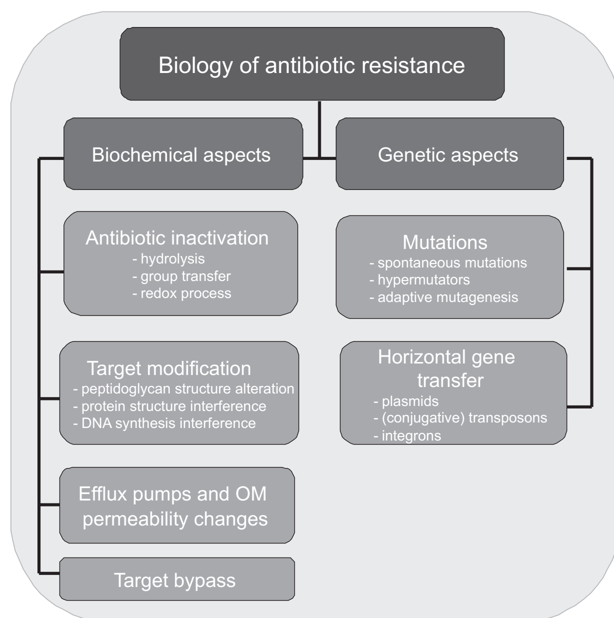

Fig. 1. Biochemical and genetic aspects of antibiotic resistance mechanisms in bacteria

direct inactivation of the active antibiotic molecule (*36*); (*ii*) Target modification – alteration of the sensitivity to the antibiotic by modification of the target (*37*); (*iii*) Efflux pumps and outer membrane (OM) permeability changes – reduction of the concentration of drug without modification of the compound itself (*38*); or (*iv*) Target bypass – some bacteria become refractory to specific antibiotics by bypassing the inactivation of a given enzyme. This mode of resistance is observed in many trimethoprim- and sulfonamide-resistant bacteria. The example is in bypassing inhibition of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes (involved in tetrahydrofolate biosynthesis). They are inhibited by trimethoprim and sulfonamides, respectively. In several trimethoprim- and sulfonamide-resistant strains, a second enzyme that has low affinity for the inhibitors is produced (*34,39*).

There is an amazing diversity of antibiotic resistance mechanisms within each of these four categories and a single bacterial strain may possess several types of resistance mechanisms. Which of these mechanisms prevails depends on the nature of the antibiotic, its target site, the bacterial species and whether it is mediated by a resistance plasmid or by a chromosomal mutation.

Antibiotic inactivation

The defence mechanisms within the category of antibiotic inactivation include the production of enzymes that degrade or modify the drug itself. Biochemical strategies are hydrolysis, group transfer, and redox mechanisms.

Antibiotic inactivation by hydrolysis

Many antibiotics have hydrolytically susceptible chemical bonds (*e.g.* esters and amides). Several enzymes are known to destroy antibiotic activity by targeting and cleaving these bonds. These enzymes can often be excreted by the bacteria, inactivating antibiotics before they reach their target within the bacteria. The classical hydrolytic amidases are the β-lactamases that cleave the β-lactam ring of the penicillin and cephalosporin antibiotics. Many Gram-negative and Gram-positive bacteria produce such enzymes, and more than 200 different β-lactamases have been identified. β-Lactamases are classified into four groups on the basis of functional characteristics, including preferred antibiotic substrate. Clinical isolates often produce β-lactamases belonging to different functional groups. They can be both chromosomal and plasmid-encoded β-lactamases transferred from different bacteria (*40–43*).

Extended-spectrum β-lactamases (ESBLs) mediate resistance to all penicillins, third generation cephalosporins (*e.g.* ceftazidime, cefotaxime, ceftriaxone) and aztreonam, but not cephamycins (cefoxitin and cefotetan) and carbapenems. ESBLs are very diverse: more than 180 different ESBLs have been identified. They are most commonly detected in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*, but have also been found in other *Enterobacteriaceae* (*44,45*). The website *http://www.lahey.org/Studies/* was established to standardize the nomenclature for the growing number of β-lactamases and provide references to sources for nucleotide and amino acid sequence information (*46*).

Other hydrolytic enzyme examples include esterases that have been linked to macrolide antibiotic resistance and ring-opening epoxidases causing resistance to fosfomycin (*47–49*).

Antibiotic inactivation by group transfer

The most diverse family of resistant enzymes is the group of transferases. These enzymes inactivate antibiotics (aminoglycosides, chloramphenicol, streptogramin, macrolides or rifampicin) by chemical substitution (adenylyl, phosphoryl or acetyl groups are added to the periphery of the antibiotic molecule). The modified antibiotics are affected in their binding to a target. Chemical strategies include *O*-acetylation and *N*-acetylation (*50–52*), *O*-phosphorylation (*53–55*), *O*-nucleotidylation (*56,57*), *O*-ribosylation (*58*), *O*-glycosylation, and thiol transfer. These covalent modification strategies all require a co--substrate for their activity (ATP, acetyl-CoA, NAD^+, UDP-glucose, or glutathione) and consequently these processes are restricted to the cytoplasm.

Antibiotic inactivation by redox process

The oxidation or reduction of antibiotics has been infrequently exploited by pathogenic bacteria. However, there are a few of examples of this strategy (*59–61*). One is the oxidation of tetracycline antibiotics by the TetX enzyme. *Streptomyces virginiae*, producer of the type A streptogramin antibiotic virginiamycin M_1, protects itself from its own antibiotic by reducing a critical ketone group to an alcohol at position 16.

Target modification

The second major resistance mechanism is the modification of the antibiotic target site so that the antibiotic is unable to bind properly. Because of the vital cellular functions of the target sites, organisms cannot evade antimicrobial action by dispensing with them entirely.

However, it is possible for mutational changes to occur in the target that reduce susceptibility to inhibition whilst retaining cellular function (*62*).

In some cases, the modification in target structure needed to produce resistance requires other changes in the cell to compensate for the altered characteristics of the target. This is the case in the acquisition of the penicillin-binding protein 2a (PBP2a) transpeptidase in *Staphylococcus aureus* that results in resistance to methicillin (methicillin-resistant *S. aureus*, MRSA) and to most other β-lactam antibiotics. To save the efficiency of peptidoglycan biosynthesis, PBP2a needs alterations in the composition and structure of peptidoglycan, which involves functioning of a number of additional genes (*39,63,64*).

Peptidoglycan structure alteration

The peptidoglycan component of the bacterial cell wall provides an excellent selective target for the antibiotics. It is essential for the growth and survival of most bacteria. Consequently, enzymes involved in synthesis and assembly of the peptidoglycan component of the bacterial cell wall provide excellent targets for selective inhibition. The presence of mutations in the penicillin-binding domain of penicillin-binding proteins (PBPs) results in decreased affinity to β-lactam antibiotics. Alterations among PBPs result in ampicillin resistance among *Enterococcus faecium*, and penicillin resistance among *Streptococcus pneumoniae* (*65–67*). Resistance to methicillin and oxacillin in *S. aureus* is associated with acquisition of a mobile genetic element called SCC*mec*, which contains the *mecA* resistance gene. The *mecA* determinant encodes PBP2a, a new penicillin-binding protein distinct from the PBPs normally found in *S. aureus.* PBP2a is highly resistant to inhibition by all clinically used β-lactams and remains active to maintain cell wall synthesis at normally lethal β-lactam concentrations (*32*).

Glycopeptides such as vancomycin inhibit cell wall synthesis of Gram-positive bacteria by binding C-terminal acyl-D-alanyl-D-alanine (acyl-D-Ala-D-Ala)-containing residues in peptidoglycan precursors. Resistance is achieved by altering the target site by changing the D-Ala-D-Ala to D-alanyl-D-lactate (D-Ala-D-Lac) or D-alanyl-D-serine (D-Ala-D-Ser) at the C-terminus, which inhibits the binding of vancomycin (*68-70*). As a consequence, the affinity of vancomycin for the new terminus is 1000 times lower than for the native peptidoglycan precursor in the case of D-Ala-D-Lac. Dissemination of glycopeptide resistance in Gram-positive cocci can occur at the level of the bacteria (clonal spread), replicons (plasmid epidemics) or of the genes (transposons). Glycopeptide (vancomycin) resistance can be intrinsic (VanC-type resistance) or acquired, present only in certain isolates belonging to the same species (VanA, B, D, C, E and G types of vancomycin resistance) (*71*).

Protein synthesis interference

A wide range of antibiotics interfere with protein synthesis on different levels of protein metabolism. The resistance to antibiotics that interfere with protein synthesis (aminoglycosides, tetracyclines, macrolides, chloramphenicol, fusidic acid, mupirocin, streptogramins, oxazolidinones) or transcription *via* RNA polymerase (the rifamycins) is achieved by modification of the specific target (*39*).

The macrolide, lincosamide and streptogramin B group of antibiotics block protein synthesis in bacteria by binding to the 50S ribosomal subunit (*72–74*). Resistance to these antibiotics is referred to as MLS(B) type resistance and occurs in a wide range of Gram-positive bacteria. It results from a post-transcriptional modification of the 23S rRNA component of the 50S ribosomal subunit (*75*). Mutations in 23S rRNA close to the sites of methylation have also been associated with resistance to the macrolide group of antibiotics in a range of organisms. In addition to multiple mutations in the 23S rRNA, alterations in the L4 and L22 proteins of the 50S subunit have been reported in macrolide-resistant *S. pneumoniae* (*76*). The mechanism of action of oxazolidinones (for example, linezolid) involves multiple stages in the protein synthesis (*77*). Although they bind to the 50S subunit, the effects include inhibition of formation of the initiation complex and interference with translocation of peptidyl-tRNA from the A site to the P site. Resistance has been reported in a number of organisms including enterococci and is linked to mutations in the 23S rRNA resulting in decreased affinity for binding (*78*).

Mutations in the 16S rRNA gene confer resistance to the aminoglycosides (*79*). Chromosomally acquired streptomycin resistance in *M. tuberculosis* is frequently due to mutations in the *rpsL* gene encoding the ribosomal protein S12. Microorganisms that produce aminoglycosides have developed mechanism of high level antibiotic resistance by posttranscriptional methylation of 16S rRNA in the aminoglycoside binding site. This mechanism of resistance has recently been reported in human pathogens from nosocomial infections and animal isolates (*80*).

DNA synthesis interference

Fluoroquinolones interact with the DNA gyrase and topoisomerase IV enzymes and prevent DNA replication and transcription. Resistance is conferred by mutations in specific regions of the structural genes that sufficiently alter these enzymes preventing the binding of antibiotics (*81,82*). The most common mutations in this region cause resistance through decreased drug affinity for the altered gyrase–DNA complex (*83–85*).

Efflux pumps and outer membrane (OM) permeability

The efflux pumps are the membrane proteins that export the antibiotics out of the cell and keep its intracellular concentrations at low levels. Reduced outer membrane (OM) permeability results in reduced antibiotic uptake. The reduced uptake and active efflux induce low level resistance in many clinically important bacteria (*86*).

Efflux pumps

Efflux pumps affect all classes of antibiotics, especially the macrolides, tetracyclines, and fluoroquinolones because these antibiotics inhibit different aspects of protein and DNA biosynthesis and therefore must be intracellular to exert their effect. Efflux pumps vary in both their specificity and mechanism (*87,88*). Although some are drug-specific, many efflux systems are multidrug transporters that are capable of expelling a wide spectrum of structurally unrelated drugs, thus contribut-

ing significantly to bacterial multidrug resistance (MDR) (*89*). Inducible multidrug efflux pumps are responsible for the intrinsic antibiotic resistance of many organisms, and mutation of the regulatory elements that control the production of efflux pumps can lead to an increase in antibiotic resistance. For example, the MexAB-OprM efflux pump in *Pseudomonas aeruginosa* is normally positively regulated by the presence of drugs, but mutations in its regulator (*mexR*) lead to the overexpression of MexAB-OprM, which confers increased resistance to antibiotics such as β-lactams (*90–92*). Both Gram-positive and Gram-negative bacteria can possess single-drug and/or multiple drug efflux pumps (*93,94*).

Bacterial drug efflux transporters are currently classified into five families (*95,96*). The major facilitator superfamily (MFS) and the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily are very large and the other three are smaller families: the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) superfamily and the multidrug and toxic compound extrusion (MATE) family. Efflux transporters can be further classified into single or multicomponent pumps (*97–99*). Single component pumps transport their substrates across the cytoplasmic membrane. Multicomponent pumps, found in Gram-negative organisms, function in association with a periplasmic membrane fusion protein (MFP) component and an outer membrane protein (OMP) component, and efflux substrates across the entire cell envelope.

Furthermore, the regulators of efflux systems may be attractive drug targets themselves. The regulators involved in efflux gene expression are either local or global regulators. Many pump component-encoding operons contain a physically linked regulatory gene. Some efflux pumps are known to be regulated by two-component systems. These systems mediate the adaptive responses of bacterial cells to their environment. Expression of various efflux pumps is also controlled by different global regulators. So far, several global transcriptional activators, including MarA, SoxS and Rob, have been shown to be involved in the regulation of expression of this system (*97–99*).

Outer membrane (OM) permeability changes

Gram-negative bacteria possess an outer membrane consisting of an inner layer containing phospholipids and an outer layer containing the lipid A moiety of lipopolysaccharides (LPS). This composition of the outer membrane (OM) slows down drug penetration, and transport across the OM is achieved by porin proteins that form water-filled channels. Drug molecules can penetrate the OM employing one of the following modes: by diffusion through porins, by diffusion through the bilayer or by self-promoted uptake. The mode of entry employed by a drug molecule largely depends on its chemical composition. For example, hydrophilic compounds either enter the periplasm through porins (*e.g.* β-lactams) or self-promoted uptake (aminoglycosides). Antibiotics such as β-lactams, chloramphenicol and fluoroquinolones enter the Gram-negative outer membrane *via* porins. As such, changes in porin copy number, size or selectivity will alter the rate of diffusion of these antibiotics (*100–104*).

The role of LPS as a barrier to antibiotics is well documented. Mutations in LPS that result in antibiotic hypersusceptibility have been reported. Strains of *E. coli* and *S. enterica* serovar Typhimurium defective in LPS have been found to be at least 4-fold more susceptible to erythromycin, roxithromycin, clarithromycin and azithromycin than the wild-type strains (*105,106*).

Genetics of Antibiotic Resistance

Studies of a wide variety of bacterial pathogens have identified numerous genetic loci associated with antibiotic resistance. For some types of resistance there is a large diversity of responsible genetic determinants.

Resistance can be an intrinsic property of the bacteria themselves or it can be acquired. Acquired bacterial antibiotic resistance can result from a mutation of cellular genes, the acquisition of foreign resistance genes or a combination of these two mechanisms. Thus, there are two main ways of acquiring antibiotic resistance: *i)* through mutation in different chromosomal loci and *ii)* through horizontal gene transfer (*i.e.* acquisition of resistance genes from other microorganisms). This raises several questions about the evolution and ecology of antibiotic resistance genes. Phylogenetic insights into the evolution and diversity of several antibiotic resistance genes suggest that at least some of these genes have a long evolutionary history of diversification that began well before the 'antibiotic era' (*107*).

Mutations

Spontaneous mutations

Exploring the origins of resistant mutants began with the antibiotic era in 1940s, when researchers performed classical experiments proving that mutations conferring resistance to certain antibiotics arise prior to or in the absence of any selective pressure. These mutation events occur randomly as replication errors or an incorrect repair of a damaged DNA in actively dividing cells. They are called growth dependent mutations (spontaneous mutations) and present an important mode of generating antibiotic resistance (*108*).

Antibiotic resistance occurs by nucleotide point mutations which are at the same time growth permissive and are able to produce a resistance phenotype (*109*). For instance, quinolone resistance phenotype in *Escherichia coli* is a result of changes in at least seven positions in the *gyrA* gene, but in only three positions in the *parC* gene (*110,111*). A variety of genes can be involved in antibiotic resistance either because there are several different targets, access, or protection pathways for the antibiotic in the bacterial cell or because each pathway requires the expression of several genes.

There is a substantial number of biochemical mechanisms of antibiotic resistance that are based on mutational events, like the mutations of the sequences of genes encoding the target of certain antibiotics (for instance, resistance to rifamicins and fluoroquinolones are caused by mutations in the genes encoding the targets of these two molecules, RpoB and DNA-topoisomerases, respectively) (*112,113*). The variation in the expression of antibiotic uptake or of the efflux systems may also be modi-

fied by mutation (for instance, the reduced expression or absence of the OprD porin of *P. aeruginosa* reduces the permeability of the cell wall to carbapenems) (*114*). Some of the resistances associated with the uptake and efflux systems are caused by mutations in regulatory genes or their promoter regions (*115,116*). Also, the mutations leading to increased expression of the efflux systems, in general, confer resistance to multiple antibiotics (for example, mutations in the *Escherichia coli mar* gene affect the expression of about 60 different genes, including down-regulation of OmpF and up-regulation of AcrAB) (*117*). AcrAB is involved in the efflux of β-lactams, fluoroquinolones, chloramphenicol, and tetracycline. In *P. aeruginosa*, mutation in *mexR* up-regulates the mexA-mexB-oprM operon and raises resistance to most β-lactams, fluoroquinolones, tetracyclines, chloramphenicol and macrolides (*118*). The overproduction of antibiotic-inactivating enzymes may also be achieved through mutational events. Many Gram-negative microorganisms produce chromosomal β-lactamases at low levels and mutations producing up-regulation of their expression may lead to the resistance to most cephalosporins.

In addition to these examples, there are some clinically relevant pathogens for which plasmid- or transposon-mediated mechanisms of resistance have not been reported (*Mycobacterium tuberculosis* isolated in the infected patients lacks the horizontal transfer mechanisms and, consequently, can acquire antibiotic resistance by mutation exclusively) (*119*). *P. aeruginosa* from the lungs of patients with cystic fibrosis is almost impossible to eradicate, mainly because of the development of resistance to multiple antibiotics. In this particular environment resistance is achieved through chromosomal mutations that are able to produce resistance to all antibiotics used in clinical practice, without any acquisition of exogenous DNA (*120*).

Hypermutators

It has been widely accepted that mutation is the unavoidable consequence of errors produced in the DNA replication process or of the failure of the error-avoidance systems. Maintaining the stability of genetic information is vital for the perpetuation of species. Low spontaneous mutation rates are maintained by the activity of many molecular mechanisms that protect and repair DNA, as well as by the mechanisms that assure high-fidelity of DNA replication (*121,122*). However, bacteria with an elevated mutation rate (hypermutable strains, or mutators) among natural and laboratory populations have been found. Experimental studies indicate that the frequency of mutators observed among natural and clinical bacterial isolates is much higher than expected, which suggests that there are situations in nature where being a mutator confers a selective advantage. According to the currently most acceptable 'hypermutable state' model, during a prolonged non-lethal antibiotic selective pressure a small bacterial population enters a transient state of a high mutation rate. If a cell in this hypermutable state achieves a useful mutation, thus relieving the selective pressure, the cell begins to grow and reproduce, and at the same time exits the hypermutable state. It is still unclear what really triggers cells to enter the hypermutable state; however, it appears that a hypermutation is regulated by a special SOS-inducible mutator DNA polymerase (pol) IV (*108*). Hypermutators have been found in populations of *E. coli*, *Salmonella enterica*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Helicobacter pylori*, *Streptococcus pneumoniae*, *P. aeruginosa* with frequencies ranging from 0.1 to above 60 % (*123, 124*).

Mutator phenotypes of different strengths could be created by inactivation of over 20 different *E. coli* genes (*125,126*). But the majority of strong mutators found in the laboratory and in nature have a defective mismatch repair (MMR) system due to the inactivation of *mutS* or *mutL* genes (*127*). This repair system controls the fidelity of DNA replication by eliminating biosynthetic errors. In addition, the mismatch repair system is involved in the maintenance of chromosomal structural integrity and in the control of horizontal gene transfer by preventing recombination between non-identical DNA sequences (*128*). The results of various studies have shown that mutators play an important role in the evolution of antibiotic resistance (*112,129–131*). By increasing the possibility of mutations, they may accelerate the evolution of favourable mutations under certain conditions. During this process, mutators can be fixed in the population by getting along with the favourable mutations (*e.g.* resistance) they have created. Thus, the acquisition of a mutator phenotype may increase the chance of acquiring antibiotic resistance by mutational events. Hypermutators may also enable multiresistant phenotype (*132*).

Adaptive mutagenesis

The mutation process has classically been studied in actively dividing bacteria, as it was assumed that most mutations occur as the consequence of errors during the DNA replication process (spontaneous mutations). However, more recent experimental data have clearly shown that mutations arise also in non-dividing or slowly dividing cells and have some relation to the selective pressure used. These mutations, named 'adaptive mutations', arise only in the presence of non-lethal selective pressure that favours them. This is the main feature that distinguishes them from the growth dependent, spontaneous mutations. The adaptive mutation process may be one of the main sources of the antibiotic resistant mutants under natural conditions (*108,133,134*). Analyses of several model systems have demonstrated that stress-enhanced bacterial mutagenesis is a regulated phenomenon (*135,136*). The main factors in this process are stress-responsive (as a part of finely regulated SOS response) error-prone DNA polymerases V (*umuCD*) and IV (*dinB*), which transiently increase the rate of mutation.

It has been demonstrated that some antibiotics (quinolones, for example) are able to induce the SOS mutagenic response and increase the rate of emergence of resistance in *E. coli* (*137*). The emergence of multiresistant strains increases in *P. aeruginosa* under antibiotic challenge (*138*). *E. coli* exposed to antibiotic streptomycin displays a hypermutable phenotype (*139*).

Some of adaptive mutations generated in mutator backgrounds (antibiotic resistance, for example) can be saved and fixed in a bacterial population either by horizontal transfer to a non-mutator background or by a reduction in the mutation rate of the adapted mutator

strain before the load of deleterious mutations becomes too high. And then, the reduction of mutation rate might be achieved by several mechanisms: reversion of the mutator allele, acquisition of suppressor mutations or by reacquisition of a wild-type allele of mutator gene from non-mutator bacteria *via* horizontal gene exchange.

Horizontal gene transfer

A principal mechanism for the spread of antibiotic resistance is by horizontal transfer of genetic material. Antibiotic resistance genes may be transferred by different mechanisms of conjugation, transformation, or transduction. Resistance genes can be further incorporated into the recipient chromosome by recombination. These genes may contain single mutations or more severe sequence changes.

Tetracycline resistance in most bacteria is due to the acquistion of new genes often associated with mobile elements. These genes are usually associated with plasmids and/or transposons and are often conjugative. The website *http://faculty.washington.edu/marilynr/*, which is updated twice a year, was established to reflect the ongoing changes in information on acquired tetracycline resistance (*tet*) and oxytetracycline resistance (*otr*) genes, originally in antibiotic producing *Streptomyces* (*140,141*). Among Gram-negative anaerobes and Gram-positive bacteria, conjugative transposons are recognized as important mediators of genetic exchange on a par with the large R-plasmids of enteric bacteria. These large (>25 kb) elements encode a fully functional conjugation apparatus and are capable of self-transfer to a wide variety of species. Conjugative transposons in the *Bacterioides* are referred to as Tc^r-elements (tetracycline resistance elements) owing to the presence of tetracycline resistance genes (*tetQ*) and these elements are primarily responsible for more than 80 % of tetracycline resistance frequency among *Bacteroides* clinical isolates (*142*). High level resistance to gentamicin and all other related aminoglycosides with the exception of streptomycin, was found in enterococci. The gene conferring this phenotype has been associated with both narrow and broad host range plasmids. The nature of these conjugative elements raises the possibility of the resistance gene spreading to other pathogenic bacteria (*143*).

Horizontal transfer of resistance genes is a mechanism for the dissemination of multiple drug resistance because resistance genes can be found in clusters and transferred together to the recipient. This is enabled by the existence of specific DNA structures called integrons (*144,145*). Integrons are DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site-specific recombination. These elements are located either on the bacterial chromosome or on broad host range plasmids. Integrons differ from transposons in two important characteristics: transposons have direct or indirect repeat sequences at their ends, but the regions surrounding the antibiotic resistance genes in the integrons are not repeats; and the integrons contain a site specific integrase gene of the same family as those found in phages but lack gene products associated with transposition. Integrons promote the capture of one or more gene cassettes within the same attachment site thereby forming clusters of antibiotic resistance genes. Gene cassettes are the smallest mobile genetic entities that can carry resistance determinants. These can encode many types of resistance including to trimethoprim, chloramphenicol, β-lactams, aminoglycosides, fosfomycin and quinolones and for each of these antibiotic classes several distinct gene cassettes have been reported. Resistance gene cassettes have been found for the most classes of antibiotics, and the gene products are involved in various mechanisms of resistance, such as efflux, target bypass and drug inactivation. Over 40 gene cassettes and three distinct classes of integrons have been identified to date (*146*).

Integron movement allows transfer of the cassette--associated resistance genes from one DNA replicon to another. Horizontal transfer of the resistance genes can be achieved when an integron is incorporated into a broad host range plasmid. A plasmid with a pre-existing resistance gene cassette can acquire additional resistance gene cassettes from donor plasmids, thus spreading multiresistance. All resistance-encoding DNAs establish a resistance gene pool, which represents a potential source for the horizontal transfer between bacteria. There are many examples of horizontal gene transfer of resistance elements both within and between bacterial species (*23, 147*). Studies about horizontal gene transfer-emerging multidrug resistance in hospital bacteria have demonstrated that the transfer of antibiotic resistance genes can take place in the intestine between Gram-positive or Gram-negative bacilli (*23*).

Multidrug-resistance (MDR) in bacteria is often the result of the acquisition of mobile genetic elements that contain multiple resistance genes. Nucleotide sequence analysis of multiresistant integrons shows that the inserted resistance gene cassettes differ markedly in codon usage, indicating that the antibiotic resistance determinants are of diverse origins (*148*). The fact that bacteria that have been separately evolving for up to 150 million years can exchange DNAs has strong implications with regard to the evolution of antibiotic resistance in bacterial pathogens (*149–151*).

Conclusion

Widespread use of antibiotics has undoubtedly caused the epidemics of antimicrobial resistance worldwide. Unfortunately, resistance in some species has developed to the level that no clinically available treatment is effective. Prevention and control strategies will require the application of epidemiological and behavioural approaches, as well as the research technologies aimed at the basic mechanisms of drug resistance. The genetic characterization of antimicrobial resistance genes as well as their location and diversity is important in identifying factors involved in resistance. It is also important to identify genetic linkages among markers and to understand potential transfer mechanisms. It appears that expression of bacterial resistance to antibiotics is frequently regulated. Modulation of gene expression can occur at the transcriptional or translational level following mutations or the movement of mobile genetic elements. It may also involve induction of mutations or gene transfer by the antibiotic so that the antibiotic can have a triple activity: as an antibacterial agent, as an in-

ducer of resistance to itself, and as an inducer of the dissemination of resistance determinants. Bacteria have elaborated mechanisms to achieve antibiotic resistance by the fine-tuning of the expression of genetic information.

Knowledge of the molecular mechanisms of antibiotic resistance is essential for developing new approaches to overcome this problem. One possible approach is the development of inhibitors of resistance enzymes. These inhibitors can be administered as co-drugs with the antibiotics, thereby blocking resistance and rescuing the antimicrobial activity of the drugs. Another strategy to overcome resistance is to improve the delivery or otherwise enhance the accessibility of antibiotics to their sites of action. For example, liposomal preparations of hydrophobic antibiotics, such as ethambutol for treatment of mycobacterial infections, have been reported. Strategies could be developed to target virulence factors of pathogens instead of whole bacteria (*e.g.* develop drugs that target the plasmids containing resistance genes or drugs that target the adhesion of virulent bacteria to a tissue).

All the alternative strategies to overcome resistance require expanded knowledge of the molecular mechanisms of antibiotic resistance, their origins and evolution, and their distribution throughout bacterial populations and genomes.

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