Douglas L. Mayers
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Editors

Antimicrobial Drug Resistance

Mechanisms of Drug Resistance, Volume 1

Second Edition

☀ Springer

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Preface

Antimicrobial drug resistance is a global health problem that continues to expand as microorganisms adapt to the antibiotics we use to treat them and as new classes of antimicrobial agents have been harder to discover and advance into the clinic. The second edition of *Antimicrobial Drug Resistance* grew out of a desire by the editors and authors to provide an updated, comprehensive resource of information on antimicrobial drug resistance that would encompass the current information available for bacteria, fungi, protozoa, and viruses. The two volumes have been extensively revised with many new authors and chapters as the field of drug resistance has evolved. We believe that this information will be of value to clinicians, epidemiologists, microbiologists, virologists, parasitologists, public health authorities, medical students, and fellows in training. We have endeavored to provide this information in a style that is accessible to the broad community of persons who are concerned with the impact of drug resistance in our clinics and across broader global communities.

Antimicrobial Drug Resistance is divided into two volumes. Volume 1 has sections covering a general overview of drug resistance and mechanisms of drug resistance, first for classes of drugs and then by individual antimicrobial agents, including those targeting bacteria, fungi, protozoa, and viruses. Volume 2 addresses clinical, epidemiologic, and public health aspects of drug resistance, along with an overview of the conduct and interpretation of specific drug resistance assays. Together, these two volumes offer a comprehensive source of information on drug resistance issues by the experts in each topic.

We are very grateful to the 197 international experts who have contributed to this textbook for their patience and support as the work came together. The editors would like to especially thank Michelle Feng He for her exceptional support and encouragement to the editors in bringing this revised textbook to print. Finally, the book would never have been completed without the patience and support of our wives and families.

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1 Introduction

Instead of eliminating infectious diseases, as some had predicted, antibiotic use has inevitably led to the emergence of more antibiotic-resistant pathogens. This chapter reviews the history of our understanding of the processes by which resistance arises. Knowledge of the chemistry and genetics of this phenomenon has allowed the development of improved antibiotics and has made major contributions to molecular biology and the biotechnical revolution.

Resistance to antimicrobial agents has been recognized since the dawn of the antibiotic era. Paul Ehrlich, the father of modern chemotherapy, observed that during treatment of trypanosome infections organisms sometimes emerged that were resistant to the agent being used. Resistance was specific in the sense that a fuchsin dye-resistant strain was still susceptible to an arsenic compound while a strain resistant to the arsenic compound retained sensitivity to the dye. He showed that resistance, once acquired, was stably inherited and in 1908 proposed that resistance was due to "reduced avidity of the chemoreceptors so that they are no longer able to take up" drug [1]. Substitute "target" for "chemoreceptor" and one of the major mechanisms for antimicrobial resistance was revealed as was its specificity for particular compounds. Drug inactivation was discovered early as well. In 1919, Neuschlosz reported that *Paramecium caudatum* resistant to quinine and to certain dyes acquired the ability to destroy the toxic agents [2].

Early on resistance was categorized as either natural or acquired. For example, natural resistance to gentian violet was a property of gram-negative as compared to gram-positive organisms. Some agents (sulfonamides, aminoglycosides, chloramphenicol, rifampin, and others) were recognized to have a broad spectrum while other agents had a narrower focus (vancomycin, macrolides, isoniazid). The less susceptible organisms were said to be naturally resistant. The natural resistance of gram-negative bacteria to dyes and many other agents was attributed to an outer membrane barrier, which with our now increased appreciation of efflux pumps is understood to be only part of the story [3]. Acquired resistance properly involved reduced susceptibility of an organism that was previously more sensitive to the drug, and was to be distinguished, if possible, from replacement of a susceptible organism by more resistant but unrelated ones, a process soon appreciated to occur all too readily in hospitals, which became breading grounds for increasingly resistant flora.

How to interpret the emergence of resistance revived a nineteenth century controversy between Nägeli and Koch. Nägeli held that microorganisms were polymorphic and could transform spontaneously in shape and biochemical behavior. Koch believed that they were monomorphic with fixed properties and hence classifiable into species that could be rigidly defined. In the 1920s and 1930s this debate took the form of belief in the influence of bacterial life cycles. The theory of microbial dissociation held that such properties as shape, nutritional requirements, antigenicity, virulence, chemical reactivity, and hence susceptibility were not fixed properties of an organism but varied with the growth phase and life cycle of the bacterial culture [4]. By this line of reasoning the appearance of antibiotic resistance was but another manifestation of dissociation.

In today's terms the issue was adaptation versus mutation. Did acquired resistance represent an adaptive response to the drug, which persisted for many generation after the drug was removed, or selection from the initial population of rare preexisting resistant mutants? The adaptation hypothesis was championed in the 1940s by Hinshelwood who argued that if a culture was grown in the presence of an inhibitor, the concentration of the substrate for the blocked reaction would accumulate and reverse the inhibition. Serial culturing in successively higher concentrations of drug was interpreted as thus "training" the culture to tolerate the inhibition [5].

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The issue was settled in favor of mutation by demonstration that resistance could emerge in the absence of antibiotic and by its transfer with DNA. For example, the Lederbergs showed by replica plating that streptomycin-resistant colonies of *Escherichia coli* were present in a culture never exposed to the drug [6], while Hotchkiss demonstrated that penicillin resistance could be transferred to a susceptible pneumococcus by DNA from a resistant one [7].

Adaptation returned later, however, in the form of adaptive mutations and adaptive antibiotic resistance. Adaptive mutations are defined as mutations formed in response to the environment in which they have been selected [8, 9]. Such mutants occur in nondividing or slowly dividing cells and are specific for events that allow growth in that environment, as, for example, the emergence of ciprofloxacin-resistant mutants in nondividing cultures of *E. coli* exposed for a week to ciprofloxacin in agar [10]. Adaptive resistance is a phenomenon seen with aminoglycosides when bacteria preexposed to the antibiotic show less killing on subsequent exposure [11]. A reappreciation of genomic plasticity returned as well as the many mechanisms of horizontal gene transfer were elucidated and again challenged the notion of fixed bacterial species.

Until penicillin became available sulfonamides were widely used for both treatment and prophylaxis, and before long resistance began to appear in several pathogens. Daily administration of sulfadiazine to prevent upper respiratory infections at military bases during World War II was followed by the emergence of resistant β-hemolytic streptococci. The question was whether the resistance was acquired or preexisting. Since the resistant organisms mainly belonged to only a few serotypes, selection of naturally resistant strains was favored although the possibility that only particular serotypes could readily acquire resistance seems not to have been considered [12, 13]. Use of sulfonamides for treatment of gonorrhea was followed by increasing failure rates and the proliferation of sulfonamide-resistant strains of *Neisseria gonorrhoeae* [14]. Increasing sulfonamide resistance was also noted in *Neisseria meningitidis* with corresponding clinical failure [15]. Whether the neisseria truly acquired resistance was unclear since sulfonamide-resistant strains were discovered in cultures of *N. gonorrhoeae* or *N. meningitidis* from the presulfonamide era [15, 16]. Sulfonamide treatment of bacillary dysentery became complicated as well by the isolation of resistant strains, especially of resistant *Shigella sonnei* [17]. Isolated instances were also reported of sulfadiazine resistance in pneumococci recovered after therapy of either pneumococcal pneumonia [18] or pneumococcal meningitis [19]. Knowledge of bacterial biochemistry and metabolism had advanced after the empirical discovery of sulfonamides so that in 1940 *p*-aminobenzoic acid (PABA) was discovered to block the action of sulfonamide. PABA was proposed to be an essential metabolite for bacteria.

Sulfonamide was hypothesized to mimic the chemical structure of PABA and to impede bacterial growth by competing with PABA to prevent its utilization [20]. Extracts of resistant pneumococci were soon found to contain increased amounts of a sulfonamide inhibitor [21], which was identified as PABA in extracts of other sulfonamide-resistant bacteria [22], so all seemed consistent with resistance as the result of PABA overproduction. The story took another twist, however, when sulfonamide-resistant *E. coli* were found to make not excess PABA but a sulfonamide-resistant enzyme that utilizes PABA in an early step of folic acid biosynthesis [23]. Such target enzyme insensitivity is now thought to be the main, if not the sole, mechanism for sulfonamide resistance [24].

The major mechanism for resistance to penicillin was much more quickly identified. The dramatic increase in penicillin resistance in *Staphylococcus aureus* that took place in the first decade of the antibiotic's use resulted from the selective advantage provided by an enzyme that inactivated penicillin, which was present initially in only a few isolates. The enzyme, penicillinase, was first described, not in *S. aureus,* but in *E. coli,* in 1940, the same year clinical studies with penicillin began [25]. By 1942 increased resistance was reported in *S. aureus* from patients receiving penicillin [26], and in 1944 penicillinase was extracted from resistant strains of *S. aureus* obtained from patients who had not even been exposed to the drug [27]. At Hammersmith Hospital in London the fraction of *S. aureus* isolates that were penicillin resistant increased rapidly from 14 % in 1946, to 38 % in 1947, and to 59 % in 1948 [28] eventually stabilizing at the 90 % resistance seen today and inspiring the development of semi-synthetic β-lactamase-resistant penicillins, which were the first antibiotics specifically designed to overcome a characterized resistance mechanism [29]. Unfortunately, methicillin-resistant *S. aureus* appeared within a few years and were found to make not a methicillin-degrading enzyme but rather a novel methicillin-resistant protein involved in cell wall biosynthesis [30, 31]. The battle between bacteria and pharmaceutical chemists synthesizing improved β-lactam antibiotics had been joined and would continue [32].

The basis of resistance to streptomycin remained a puzzle for a long time. Streptomycin-resistant mutations arose at low frequency in many kinds of bacteria, including, unfortunately, *Mycobacterium tuberculosis* when the agent was used alone for treatment. Mutation produced not only high-level resistance but also bacteria dependent on streptomycin for growth, a curious type that could even be recovered from patients treated with the drug [33]. A variety of biochemical changes followed exposure to streptomycin, including damage to the cell membrane [34], but it was the observation that growth of a streptomycin-dependent mutant of *E. coli* in a suboptimal concentration of streptomycin resulted in

decreased concentrations of protein and increased amounts of RNA that led Spotts and Stanier to propose that streptomycin blocked protein synthesis in susceptible cells but was required for proper mRNA attachment to the ribosome in dependent ones [35]. Direct demonstration that streptomycin impaired amino acid incorporation in a cell-free system soon followed [36]. Streptomycin at a concentration as low as l0⁻⁶ M could inhibit polyuridylate directed incorporation of phenylalanine, but a 1000-fold higher concentration was required if the cell-free system was derived from a streptomycin-resistant organism. Furthermore, streptomycin was found to cause misreading of the genetic code so that in its presence polyuridylate catalyzed the misincorporation of isoleucine and other amino acids [37]. So much was learned in studying the interaction of streptomycin and other drugs with the bacterial ribosome [38] that it came as something of a surprise that clinical isolates resistant to streptomycin relied on quite a different strategy, namely modification by adenylation, phosphorylation, and, for other aminoglycosides, acetylation as well [39]. The lesson that resistance selected in the laboratory could be different from that selected in the clinic had to be learned.

Resistance to other antimicrobial agents emerged and was studied, but the next major conceptual advance was the appreciation of the importance of R-plasmids, which led not only to a better understanding of resistance acquisition and dissemination but ultimately to recombinant DNA and the biotechnology revolution. The demonstration of transferable resistance in Japan dated from 1959 but took several more years to attract attention and be accepted [40, 41]. An explosion of discoveries followed. R-plasmids were found around the world not only in *Enterobacteriaceae* but also in pseudomonas, acinetobacter, staphylococci, enterococci, bacteroides, clostridia, and in virtually every bacterial species examined. Some had remarkably wide host ranges while others were limited to gram-positive, gram-negative, anaerobic, or even smaller bacterial subsets. Techniques were developed for plasmid transfer, isolation, and classification [42, 43]. Transposons that allowed resistance genes to jump from one DNA site to another were discovered [44], as were integrons that allowed resistance gene cassettes to be captured on plasmids and efficiently expressed [45], and specialized insertion sequences adept at gene capture [46]. Restriction enzymes, often plasmid-mediated, facilitated analysis of plasmid structure and permitted DNA cloning. The genetics of antibiotic resistance became as tractable as its biochemistry and contributed much to the emerging discipline of molecular biology.

The finding that a β-lactamase (designated TEM) from a clinical isolate of *E. coli* was carried on an R-plasmid [47] led to the realization that this resistance mechanism could spread not only to other *E. coli* but also to other genera. Before long TEM β-lactamase was found in ampicillin-resistant *Haemophilus influenzae* [48] and in penicillin-resistant *N. gonorrhoeae* [49]. Enzymes more active on cephalosporins than penicillins were discovered, functional classification of the growing body of β-lactamases began [50], the technique of isoelectric focusing was added to the repertoire of β-lactamase biochemists [51], introduction of cefamandole led to the recognition that β-lactamase derepression could provide resistance in some organisms [52], and clinical use of expanded-spectrum cephalosporins was followed by an explosion of extended-spectrum and other β-lactamases [32, 53].

Plasmids carry genes for resistance to many other antimicrobial agents. Some genes code for enzymes that modify or inactivate the agents, others for enzymes that alter drug targets in the cell or provide alternate biosynthetic pathways. Genes for antibiotic efflux (chloramphenicol, tetracycline) were also found to be plasmid-determined, but efflux-mediated resistance occurred as well from chromosomal mutations that alter control circuits also involved in expression of outer membrane proteins that form porin channels for antibiotic uptake. Study of bacteria collected in the preantibiotic era indicated that the plasmids that organize, express, and transmit resistance predated the clinical use of antibiotics [54]. R-plasmids resulted from the insertion of resistance genes into previously existing vehicles for their spread. The resistance genes themselves have had a diverse origin. Some have come from organisms producing antibiotics since these organisms needed a mechanism for self-protection [55, 56]. Others are now appreciated to have been present in environmental organisms for millennia to counteract the biological weapons of competing antibiotic producers. Potential reservoirs of resistance genes have been found in ancient permafrost and at the bottom of caves sealed from above for millions of years [57, 58].

Plasmids are not the only vehicle for gene transfer. Naturally transformable pathogens such as *Streptococcus pneumoniae, N. meningitidis, N. gonorrhoeae,* and *H. influenzae* were found to exchange chromosomal genes with members of closely related species, including genes for penicillin-binding proteins and topoisomerases that provide resistance to penicillin or quinolones [59–61]. Mutation plays an important role in resistance to some antimicrobial agents usually by altering enzyme specificity or reducing binding to a lethal target. The notion that resistance was based on infrequent mutational events also led to the concept that resistance could be prevented by simultaneous administration of two drugs since the product of the likelihood of resistance emerging to each would be greater than the size of any possible infecting inoculum, a thesis best justified by the success of multidrug treatment of tuberculosis. An increased mutation rate eventually exerts a fitness cost, but limited rate increases have been found in organisms with resistance attributable to an altered target

(quinolone resistance from *gyrA* mutations) [62] or modified enzyme (expanded-spectrum β-lactam resistance due to extended-spectrum β-lactamases) [63].

Antibiotic resistance has come to be accepted as an inevitable consequence of antibiotic use. The ubiquity of the phenomenon has been amply illustrated with emerging resistance to antiviral, antifungal, and anti-parasitic agents as well. On the positive side understanding the mechanisms of antibiotic resistance has often provided important insights into how antibiotics work. Knowledge about R-factors has unfortunately not made a direct attack on the genetic basis of resistance possible, but insight into resistance mechanisms has guided the development of expanded-spectrum β-lactams (cefepime, cefotaxime, ceftazidime, ceftriaxone, aztreonam, and others), aminoglycosides (amikacin, dibekacin, arbekacin, plazomicin, and others), and tetracyclines (tigecycline) as well as currently available β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) and others undergoing evaluation (avibactam). A number of enigmas remain. Some organisms, such as *S. aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa,* seem particularly adept at acquiring resistance while others are puzzlingly reluctant with certain drugs. *Treponema pallidum* and *Streptococcus pyogenes*, for example, remain fully susceptible to penicillin G despite decades of exposure to the drug while other organisms have become progressively more resistant. The tempo at which resistance develops is also remarkably variable (Table 1.1). Resistance may appear soon after a drug is introduced or only after many years. Methicillin-resistant *S. aureus* were isolated in the United Kingdom within a few years of the drug being introduced [64, 65], but 20 years elapsed before pneumococci with reduced susceptibility to penicillin were isolated and another 20 years before resistance was recognized as a worldwide problem [66]. Vancomycin resistance took even longer to appear [67]. The equilibrium level at which resistance

becomes stabilized is also curiously variable. β-Lactamase production has reached 10–30 % in the gonococcus, 15–35 % in *H. influenzae*, 30–40 % in *E. coli,* 75 % in *Moraxella catarrhalis*, and 90 % in *S. aureus*, but what determines these levels is poorly understood. Once it has been acquired, however, resistance is slow to decline [68] and there are few examples of reduced antibiotic use associated with diminished resistance [69] so that prevention of resistance by prudent antibiotic use remains the keystone to control. Appropriate use applies as well to nonhuman applications with restraining antibiotics in animal feed a prominent example.

Table 1.1 Timetable of antibiotic discovery and resistance

Antibiotic	Discovered or reported	Clinical use	Resistance identified	Organism
Sulfonamide	1935	1936	1939	*S. pneumoniae*
Penicillin G	1928	1941	1942	*S. aureus*
	1940 (purified)		1965	*S. pneumoniae*
Methicillin	1960	1960	1961	*S. aureus*
Oxyimino-β-lactams	1978	1981	1983	*K. pneumoniae*
				E. coli
Streptomycin	1944	1946	1946	*E. coli*
Tetracycline	1948	1952	1959	*S. dysenteriae*
Erythromycin	1952	1955	1957	*S. aureus*
Vancomycin	1956	1958	1987	*E. faecium*
Gentamicin	1963	1967	1970	*K. pneumoniae*
				P. aeruginosa

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Evolutionary Biology of Drug Resistance

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1 Introduction

It is widely upheld that evolution is the result of two essential forces: variability (chance) and selection (necessity). This assumption is confirmed by a number of simple phenomena in antibiotic resistance. Variability is created by random mutation (also recombination), and some of these variants (for instance, those with a mutation in the antibiotic target) become resistant. These variants are selected by antibiotic use and consequently they increase the frequency of resistance. If we increase variability (as in a hyper-mutable strain) or the intensity of selection (antibiotic hyper-consumption), the result is more resistance. This is true, but not the whole truth. Most determinants of antibiotic resistance are not based on simple mutations, but rather on sophisticated systems frequently involving several genes and sequences; moreover, resistance mutations are seldom transmitted by lateral gene transfer. The acquisition of any type of resistance produces a change. In biology, any change is not only an opportunity, but is also a risk for evolution. Bacterial organisms are highly integrated functional structures, exquisitely tuned by evolutionary forces to fit with their environments. Beyond the threshold of the normal compliance of these functions, changes are expected to disturb the equilibrium. Therefore, the acquisition of resistance is not sufficient to survive; evolution should also shape and refine the way of managing resistance determinants. Under the perspective of systems biology, this biological

dilemma is presented as "evolvability versus robustness", where only robust systems (able to tolerate a wide range of external changes) survive, but in the long term they should reorganize their compositional network so that they can address new and unexpected external changes. In fact, we can expect a constant cycle between robustness and evolvability in antibiotic resistance, which is manifested by changes in the frequency of some particular resistant clones.

Indeed, the field of research in drug resistance is becoming more and more complex, and constitutes a growing discipline. More than 40 years ago, Yves A. Chabbert (a brilliant pioneer in research about resistance) and one of us (F.B.) asked the pharmacologist John Kosmidis to coin the right Greek expression to describe "the science of studying resistance", and he immediately produced the word "antochology" (from Αντοχυ, resistance). To our knowledge, it was not used before the publication of the first edition of this book in 2009. In this chapter, we will examine the concept of resistance genes, the effectors of antibiotic resistance, and two essential processes that shape microbial evolution of drug resistance. First, **variability**, the *substrate of evolution*, the process providing material in evolutionary processes. Second, **selection**, the *mechanism of evolution* [1], the process by which evolution is able to adapt genetic innovation to environmental needs in the bacterial world. These evolutionary processes are embedded in a complex hierarchical network of interactions involving population dynamics of the biological elements involved in resistance, from particular genetic sequences, to genes, operons, mobile genetic elements, clonal variants, species, consortia of microorganisms, microbiotas, hosts and their communities, and the environment.

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2 Resistance Genes, the Effectors of Antibiotic Resistance

Resistance genes are those that produce a protective or adaptive effect in a microorganism in response to the deleterious input following exposure to anthropogenic antimicrobial

agents. Note that implicitly this definition contains the concept that, in a strict sense, antibiotic resistance is resistance to antibiotic therapy, that is, resistance as a threat for public health and consequently for the patient and for human population. It is true that there are differences in antibiotic susceptibility among different bacterial organisms, but certainly "bacteria were not born susceptible"; by reasons totally unrelated with antibiotic exposure, many bacterial organisms are unsusceptible or poorly susceptible to some antimicrobial agents. For instance, *Escherichia coli* is "resistant" to macrolides, only because the structure (lipopolysaccharides-based) and function (physiological pumps, such as AcrAB) of the *E. coli* outer membrane do not allow these drugs to reach in sufficient quantity at the otherwise "susceptible" ribosomal targets. Obviously the genes encoding for the outer membrane cannot be considered antibiotic "resistance genes", and "resistance" can be considered here as a "false phenotype". However, if genes involved in lipopolysaccharide or AcrAB pumps are functionally eliminated, *E. coli* become more susceptible to macrolides, but that does not make them "resistance genes". In fact bacterial cells of all species contain a large number of genes (may reach 1 % of the genome) whose knock-out (or eventually mutations) or hyper-expression results in a decrease in susceptibility to antimicrobial agents. These genes constitute the "intrinsic resistome" for a given bacterial species [2]. The "natural resistance" or "intrinsic resistance" of particular species to certain antibiotics depends on these genes, which are normally part of the bacterial chromosome "core" genome, involved in the physiological functions of the cell.

Metagenomic studies have identified many of these genes as "resistance genes", and are inappropriately included as such in databases. As frequently new "resistance genes" are defined by homology with existing genes, the noise in databases may increase exponentially. Most of the mistakes in such attribution are related with three groups of genes: (1) genes belonging to the intrinsic resistome, (2) genes encoding antibiotic targets harbouring particular mutations, and (3) genes with insufficient degrees of genetic identity with resistance genes of clinical importance.

However, we cannot fully exclude that some of these genes could act as "true" resistance genes when they enter in another (susceptible) organism exposed to antibiotics. In their original host, these genes perform physiological functions, and are generally inserted in a functional network. Out of the original host, decontextualized genes might be selected as true resistance genes. The first condition for this is that these genes could be captured by mobile genetic elements (MGEs). Second, the bacteria harbouring resistance genes in MGEs should have sufficient genetic and ecologic connectivity with bacteria able to produce infections in humans. Third, that these genes encode for resistance to relevant antibiotics used in the therapy of infections, more so if these antibiotics were not known to be detoxified by other mechanisms. Considering these main factors, the different resistance genes that might be found in metagenomic resistomes can be classified into different levels of risk for health [3, 4].

3 Variability: The Substrate of Evolution of Drug Resistance

3.1 The Complexity of Antibiotic Action and the Variety of Resistance Phenotypes

The classic dominance of either mechanistic or clinical thought in microbiology has oversimplified the image of the possible harmful consequences of exposure to industrially produced antibiotics in the microbial world. From this point of view, antibiotics are considered as *anti-biotics*, anti-living compounds found or designed to either stop the growth or kill bacterial organisms. Their main molecular targets have been identified. Nevertheless, recent studies on sub-inhibitory effects of antibiotics demonstrate that the effects of antibiotic exposure in bacteria are much larger, and therefore the adaptive and evolutionary consequences of their action are also much more complex. First, at the cellular level, the effect of antibiotic exposure is not confined to the inhibition of a single lethal target and may cause secondary effects on bacterial metabolism. Second, at the population level, the effect of antibiotic exposure is not confined to the local extinction of a harmful bacterial organism. Antibiotics exert actions on the individual cells at concentrations far lower than those needed to inhibit growth or kill bacteria.

Recent studies of gene expression suggest that a number of cellular functions (some of them increasing fitness) are modified when bacteria are exposed to sub-inhibitory concentrations of antibiotics [5, 6]. Sub-inhibitory concentrations of aminoglycoside antibiotics induce biofilm formation in *Pseudomonas aeruginosa* and *E. coli*. In *P. aeruginosa*, the aminoglycoside response regulator gene (*arr*) is essential for this induction and has contributed to biofilm-specific aminoglycoside resistance [7]. These results support the notion that antibiotics in nature are not only bacterial weapons for fighting competitors, but they are also signalling molecules that may regulate the homeostasis of microbial communities. Competition, in microbial communities, is seldom a permanent effect; competitors might just be sufficiently aggressive to control the size of their populations, in order to avoid dominance of a single genotype. Diversity, rather than dominance of a particular group, is the hallmark of evolutionary success. Indeed the major aim of evolution is to survive, to persist in time; finally, the gain in space or in cell numbers only serves to assure persistence in time [8]. This view about an ecological role of antibiotics, serving as both weapons and signals (the classic armament-ornament duality) should immediately influence our view about the evolution of resistance traits [5]. If antibiotics act as weapons in nature, antibiotic resistance develops not only to prevent

Table 2.1 Levels of specificity in antibiotic resistance

- Target mutation or alternative target production
- Inducible enzyme protecting target
- Constitutive enzyme protecting target
- Inducible enzyme detoxifying the antibiotic
- Constitutive enzyme detoxifying the antibiotic
- Rewiring of physiological systems altered by antibiotic exposure
- Mutation in specific mechanism for antibiotic uptake
- Inducible efflux system
- Constitutive efflux system
- Alterations in general mechanisms of antibiotics uptake
- Nonspecific envelope permeability alterations
- Global stress adaptive responses
- Phenotypic tolerance related with cell cycle
- Environment-dependent resistance

suicide in the producer organisms, but also to protect the diversity of the coexisting microbial communities. If in natural environments the weapons are intended to be just sub-lethal, just to modulate the growth rate or to alter the gene expression profile of microbes sharing the same habitat, resistance traits are modifiers or back-modulators of these effects. Indeed we should be open to consider that the emergence and evolution of resistance not only applies for high-level, clinically relevant resistance, but also for resistance protecting the modulation of microbial interactions. If these interactions are important to maintain the bacterial lifestyle, resistance will develop even at very low "signalling" concentrations. In short, there are a multiplicity of effects of antibiotics in bacteria; consequently, there are many levels on which antibiotic resistance is exerted, from very specific to very general ones (Table 2.1).

3.1.1 Adaptation Without Change: Redundancy and Degeneracy of Bacterial Systems

Even though antibiotics might exert a number of effects on the bacterial cell even at low antibiotic concentrations, a number of cells within a population will be essentially unaffected and could restore the original population (see also "phenotypic tolerance" in the next Sect. 3.1.2). At biological system level, this is an example of environmental *canalization* defined as the property of a biological system to maintain the normal standard phenotype despite environmental perturbations. This *robustness* or inertia to perturbation depends in part on the redundancy and degeneracy of the biological system. *Redundancy* means that multiple identical units perform the same or very similar functions inside the system. For instance, by assuring high reproductive rates, which results in high cell densities, the negative effects of variation on the entire population is diluted. Indeed small populations have a high risk of extinction by deleterious variation. Interestingly, bacteria tend to increase their replication rate at concentrations of growth-inhibiting substances that are only slightly lower than those that prevent multipli-

cation, but the adaptive impact of this phenomenon has as yet been scarcely explored.

If a number of individuals are lost after a challenge, many other almost-identical individuals are available to replace them, thus repairing the system. Note that the reconstruction of the population depends on a relatively low number of individuals, and therefore the new population will be purged to some degree of its original genetic diversity (periodic selection). At higher complexity levels, degenerate individuals may also compensate for losses in units within a system. *Degeneracy* means that structurally different units can perform the same or very similar functions in the system. Probably clonal diversification can be viewed as a way of increasing degeneracy within bacterial species. In short, redundancy and degeneracy tend to prevent antibiotic-mediated disordering events in high-level complexity bacterial systems, and lead to highly optimized tolerance. In the bacterial world, as redundant individuals are disposable they may be imported by other similar systems under danger of disorder. Hence, we can add *connectivity*—the ability of elements and systems to interact—as a means for increasing such tolerance.

3.1.2 Phenotypic Tolerance

Non-inherited antibiotic resistance (non-susceptibility) illustrates the flexibility of bacterial populations to adapt to antibiotic challenges. As stated in the previous paragraph, fully susceptible bacteria from the genetic point of view (that is, lacking specific mechanisms of resistance) might exhibit phenotypic tolerance to antibiotics, that is, they are able to persist at concentrations in which the majority of the population is dying. Cells regrown from these refractory bacteria remain as susceptible to the antibiotic as the original population [9]. Although canalization, redundancy, and degeneracy probably contribute to this phenomenon, it is the changes in the physiological state of bacterial organisms along the cell cycle that are probably critical. In practical terms, the main trait of the phenotype is slow growth. Experiments have shown that when growing bacteria are exposed to bactericidal concentrations of antibiotics, the sensitivity of the bacteria to the antibiotic commonly decreases with time and substantial fractions of the bacteria survive, without developing any inheritable genetic change [10]. Interestingly, these tolerant subpopulations generated by exposure to one concentration of an antibiotic are also tolerant to higher concentrations of the same antibiotic and can be tolerant to other types of antibiotics. It is possible that in any bacterial population, a certain spontaneous switch might occur between normal and persister cells, and it has been proposed that the frequency of such a switch might be responsive to environmental changes [11]. Such switching is probably stochastic, and depends on the random induction of persister cells through the activation of the alarmone (p)ppGpp resulting in increasing function of mRNA endonucleases [12]. In fact, we could designate as "persistence" the result of such a

switch, and phenotypic tolerance or indifference to drugs as the physiological status of any cell to become refractory to drugs. However, in our opinion such distinctions are not always clear. Mathematical modelling and computer simulations suggests that phenotypic tolerance or persistence might extend the need of antibiotic therapy, cause treatment failure of eradication, and promote the generation and ascent of inherited, specific resistance to antibiotics [13].

3.2 The Source of Antibiotic-Resistance Genes

Genes currently involved in antibiotic-resistance may have evolved for purposes other than antibiotic resistance (Table 2.2). From this point of view, resistance should be considered as a chance product, determined by the interaction of an antibiotic and a particular genotype. This is not incompatible with the idea of a gradual modification of some genes of pre-existing cellular machinery to finally "convert" into resistance genes. Some genes which may be neutral or almost neutral in the prevailing non-antibiotic environment may possess a latent potential for selection that can only be expressed under the appropriate conditions of antibiotic selection. In this case we are probably facing a *pre-adaptation* [14, 15], in the sense of assumption of a new function without interference with the original function via a small number of mutations, or gene combinations. In a later paragraph we will see in details the possible origin of enzymes hydrolyzing beta-lactam antibiotics (beta-lactamases) as an alteration of the tridimensional structure of the active site of cell wall biosynthetic enzymes (transglycosylases-transpeptidases). In other cases, the mere amplification of genes with small activity for the purposes of resistance may also result in a resistant phenotype [16]. Finally, we can have an *exaptation* [17] if the genetic conditions which exist for a function are equally well adapted to serve for antibiotic resistance.

A reservoir of "unknown" resistance genes in the intestinal microbiome has been suggested [18] even though a number of these genes have not been functionally confirmed (might have structural resemblance with resistance genes, but the resistance function was not proven). Cryptic beta-lactamase-mediated resistance to carbapenems is present in intestinal *Bacteroides* or in *Listeria* [19–21]. Metallo-beta-lactamases (MBLs) can be found in the genomes of 12 different Rhizobiales [18]. Fifty-seven open reading frames were classified as potential MBLs. Four of them were functionally analysed and one was demonstrated to be a functional MBL. Broad-spectrum chromosomally mediated beta-lactamases are usually found in Gram-negative organisms. Quinolone-resistance *qnr* genes, now plasmid-mediated, were originated in the chromosome of aquatic bacteria, such as *Shewanella algae* [22, 23]. Cryptic tetracycline-resistance determinants are present in the chromosomes of susceptible *Bacillus*, *Bacteroides*, or *E. coli*

strains as well as aminoglycoside modifying enzymes in some Enterobacteriaceae species and *P. aeruginosa* . Resistance mediated by drug-efflux pumps constitutes an excellent example of exaptation. For instance, a blast search for proteins similar to the macrolide-resistance Mef protein of *Streptococcus* reveals hundreds of hits of similar sequences encompassing all microorganisms, including *Neisseria, Bacteroides, Legionella, Enterococcus, Desulfitobacterium, Lactococcus, Lactobacillus, Ralstonia, Bacillus, Geobacter, Thermologa,* or *Streptomyces*. More recently, the possibility that genetic variants of the aminoglycoside-inactivating enzyme *aac*(6′)-*Ib* gene might reduce the susceptibility to quinolones was reported [22]. A number of these enzymes are normal chromosomal genes in a number of species, such as members of Enterococci, where they can contribute to so-called *natural resistance* to aminoglycosides and quinolones. Clinical resistance to aminoglycosides is also due to target modification by A1408 16SrRNA methyltransferases, which have been found in environmental Actinobacteria and Firmicutes [24].

The evolution of vancomycin-resistance multigene determinants is particularly intriguing. They are found in a limited number of complex operon-clusters. However these clusters are composed of genes from different sources, and almost certainly originated from a genus other than *Enterococcus*, such a *Bacillus* and *Paenibacillus* for *vanA, Clostridium, Atopobium, or Eggerthella* for *vanB,* that is, environmental aerobic or strict anaerobic bacteria from the bowel flora. The classic "**eye evolution problem**" applies here. It is difficult to conceive how such a complicated mechanism of defence against glycopeptidic antibiotics might have evolved, as apparently all its intricate functions are required for the vancomycin-resistance phenotype. In the case of the many different elements that are needed to "construct" an eye, a principal component should emerge first (in the eye, the starting point is the existence of light-sensitive cells). Some small degree of glycopeptide resistance must have evolved first (probably mediated by D-Ala:D-lac ligases) and this must have been selected and eventually refined by further evolutionary steps, that certainly include the modular recruitment of genes with functions primarily unrelated with antibiotic resistance, as two-component stimulus–response coupling (sensing-transcription) mechanisms. Without this inducible mechanism there is in fact a drastic reduction in the levels of resistance to beta-lactam antibiotics and vancomycin [25]. It is likely that unsuccessful combinations have been produced along time, and probably a number of different "solutions" have arisen. Indeed photoreceptors or eyes have also independently evolved more than 40 times in the animal kingdom. This example illustrates how nature evolves in many parallel ways, and the same occurs for drug resistance. The high diversity in determinants of resistance strongly suggests that many of them have evolved to the current function from "pre-resistance" molecules originated