Douglas L. Mayers
Jack D. Sobel · Marc Ouellette
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Editors

Antimicrobial Drug Resistance

Mechanisms of Drug Resistance, Volume 1

Second Edition

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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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Part I

General Overview

History of Drug-Resistant Microbes

1

George A. Jacoby

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 pre-existing 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 pre-exposed 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 10^{-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

2

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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.

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

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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 sublethal, 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 multiplication, 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 intrincate 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

Table 2.2 Examples of resistance mechanisms in clinical strains that evolved from natural functions in non-clinical organisms

Antibimicrobial group	Mechanisms	Related natural protein	Natural reservoirs
Aminoglycosides	Acetylation	Histone-acetylases	*Streptomyces*
	Phosphorylation	Protein kinases	*Actinobacteria, Firmicutes*
	16S rRNA methyltransferases	The same	
Tetracyclines	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	*Streptomyces*
Chloramphenicol	Acetylation	Acetylases	*Streptomyces*
	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	
Macrolides	Target site modification	rRNA methylases	*Streptomyces*
ß-lactams (methicillin)	PBP2a	Homologous PBP2a	*Staphylococcus sciuri*
ß-lactams (cefotaxime)	CTX-M-3 beta-lactamase	Homologous beta-lactamases	*Kluyvera ascorbata*
ß-lactams (carbapenems)	OXA-48 like beta-lactamase	Homologous beta-lactamases	*Shewanella xiamenensis*
Glycopeptides (vancomycin)	Target site modification: D-ala-D-ala replacement (Van operon)	Van operon homologous genes	*Paenibacillus, Streptomyces, Amycolatopsis*
Fluoroquinolones	Topoisomerase protection	Qnr like protein	*Shewanella algae*
	Topoisomerase protection	QnrS like protein	*Vibrio splendidus*
	Efflux	QepA protein	*Streptomyces*

from different evolutionary lineages. Indeed we know about dozens of aminoglycoside-modifying enzymes, thousands of beta-lactamases, many of them redundantly inactivating the same antibiotic substrates.

This panorama helps to visualize the almost unlimited number and variety of potential antibiotic-resistance determinants in the microbial world. Because most bacterial pathogens enter periodically or are hosted in the environment, research on antibiotic resistance should be placed in the field of environmental microbiology [26, 27]. Many of the ancestor or current genes involved in actual or potential mechanisms of resistance are located in environmental bacteria. In a particular location, the ensemble of all these resistance genes constitutes the local **resistome** [28, 29]. The size of the environmental resistome can be determined by metagenomic technology dissecting local microbiomes, using gene-capture platforms particularly sensitive for the detection of resistance genes along with recent bioinformatic approaches for data mining and metagenomics.

Antibiotic-producing microorganisms might still be considered as a suitable source of highly efficient resistance determinants. It can be presumed that both antibiotic biosynthetic pathways and the mechanisms of resistance avoiding self-damage may be the result of a co-evolutionary process. In fact, resistance can be viewed as a pre-condition for significant antibiotic production. The benefit associated with antibiotic production (probably preventing habitat invasion by sensitive competitors) [30] probably also selected the producer strains harbouring the more efficient resistance strategies. As previously stated before, these resistance mechanisms may in their turn have originated in housekeeping genes (for instance, sugar kinases or acetyl-transferases for aminoglycoside resistance) [31, 32] (Table 2.1).

At closer evolutionary times, it is undeniable that most of the current mechanisms of antibiotic resistance might be derived from commensal organisms of the normal microbiota of human and animals, after older exchanges with environmental organisms. Because of that, research on antibiotic resistance forms part of the "One Health" approach, encompassing humans, animals, and the environment [33].

3.2.1 Origin of Drug Resistance: The Case of Beta-Lactamases

The origin and function of beta-lactamases in nature are still a matter of debate. Current knowledge upholds that PBPs and beta-lactamases are related to each other from a structural and an evolutionary point of view and that these proteins might have common ancestors in primitive antibiotic producer bacteria [34]. Certainly, at their turn, both beta-lactamases and PBPs should derive from ancient carboxypeptidases. It has been traditionally postulated that antibiotic-producing bacteria need to produce their own antidote to avoid committing suicide and that beta-lactam and beta-lactamase production in these organisms could be co-regulated. The filamentous soil bacteria such as *Streptomyces, Nocardia*, and *Actinomadura* produce, among others, beta-lactam antibiotics and beta-lactamases and soil fungi such as *Penicillium* are also able to produce beta-lactam antibiotics. Some of the genes participating in the biosynthesis of beta-lactams, such as *cef* or *pcb* gene variants, share similar sequences in different species of antibiotic producers, including *Cephalosporium*, *Streptomyces,* and *Penicillium*. Amino acid sequence alignment and bioinformatic analysis led to the proposal that all these genes have evolved from an ancestral gene cluster that was later mobilized from ancient bacteria to pathogenic organisms. Horizontal gene transfer must have taken place in the soil about 370 million years ago and multiple gene transfer events

occurred from bacteria to bacteria or bacteria to fungi [35]. Beta-lactam gene clusters participating in antibiotic biosynthesis also often include genes for beta-lactamases and PBPs. The beta-lactamase gene products have been shown to participate in part in the regulation of the production of these antibiotics such as cephamycins in *Nocardia lactamdurans* or cephalosporin C in *Streptomyces clavuligerus*. The latter also produces a potent inhibitor of class A beta-lactamase, probably to protect itself from formed antibiotics.

Beta-lactamases and PBPs also share issues other than potential common ancestors, gene sequences, or potential involvement in antibiotic biosynthesis regulation. Both of them have functions in relation to cell wall and peptidoglycan, which are more evident in the case of PBPs. These proteins are responsible for assembly, maintenance, and regulation of peptidoglycan structure. They are mainly anchored in the bacterial inner membrane, with their active site in the periplasmic space in Gram negatives and the corresponding space in Gram positives. In parallel, most of the beta-lactamases are secreted to the periplasmic space in the Gram negatives or evade the peptidoglycan barrier in the Gram-positive organisms. All PBP classes, with the exception of one which appears to be Zn^{2+} dependent, and beta-lactamase classes are serine active site proteins (see below). Peptidoglycan degrading products can regulate the production of beta-lactamases in certain Gram-negative bacteria due to the action of PBPs or beta-lactam antibiotics. In contrast, natural chromosomal beta-lactamases in these organisms have been shown to participate in the regulation of precursors of peptidoglycan.

Amino acid sequence analysis of PBPs and beta-lactamases argue in favour of a common origin of these proteins. Both proteins are members of a single superfamily of active-serine enzymes that are distinct from the classical serine proteases. The amino acid alignments of the main PBPs and different beta-lactamases reveal the presence of conserved boxes with strict identities or homologous amino acids. Moreover, site-directed mutagenesis in the residues essential for the catalytic activity of PBP in *E. coli* and the counterpart residues in class A beta-lactamases has shown similar features in these positions. In essence, the same structural motifs that bind penicillin in PBPs can be used to hydrolyze beta-lactams for beta-lactamases [36].

Structural evidence also supports the proposal that beta-lactamases descend from the PBP cell wall biosynthesis enzymes [37]. PBPs are ancient proteins as bacteria came into existence approximately 3.8 billion years ago, but the development of beta-lactamases is a relatively recent event, which must have taken place after the evolution of the first biosynthetic pathway in beta-lactam-producing organisms. It has been argued that this process has been reproduced several times to generate the different class A, C, and D beta-lactamases. Beta-lactamases have had to undergo structural alterations to become effective as antibiotic resistance enzymes, avoiding the interaction with the peptidoglycan or peptidoglycan precursors, which are the substrates for PBPs. This has been disclosed in X-ray interaction models with cephalosporin derivatives and AmpC beta-lactamase variants from *E. coli*. These models revealed not only three dimensional structural similarities but also that the surface for interaction with the strand of peptidoglycan that acylates the active site, which is present in PBPs, is absent in the beta-lactamase active site. The possible mutational pathways of evolution from PBPs to beta-lactamases have been investigated [38], but certainly this process might have evolved separately, by mutation and/or recombination, on many occasions.

Alternative hypotheses of the origin and function of beta-lactamases have also been postulated. Antibiotics are known to be secondary metabolite compounds that are normally released in the early stationary growth phase. For this reason, it has been hypothesized that beta-lactamases may also play a role as "peptidases", in catalysing the hydrolysis of the beta-lactam nucleus to reutilize carbon and nitrogen as an energy source in adverse conditions and they may act as nutrients for potential growing bacteria [39]. Some environmental organisms, including some *Burkholderia cepacia* genomovars and *Pseudomonas fluorescens,* have been shown to grow in the presence of penicillin as a sole carbon and nitrogen source and to stimulate the synthesis of beta-lactamase under this condition. From an evolutionary point of view, the beta-lactamase-producing bacteria have had advantages over non-beta-lactamase-producing organisms, particularly in soil communities. The former have been able not only to avoid the action of natural beta-lactam products secreted by these antibiotic producers but also to simultaneously use beta-lactams as nutrients.

3.3 Global Stress Regulation and Antibiotic Resistance

In most cases, antibiotic resistance requires time to be expressed in a particular bacterial cell. The best example is when this expression occurs as a consequence of antibiotic exposure (antibiotic-mediated induction). Only bacteria able to survive during the time required for full induction of resistance mechanisms will be able to resist antibiotic effects and consequently be selected. This "need-to-resist-to-become-resistant" paradox deserves some explanation. Antibiotic action, even at sub-inhibitory conditions, results in alterations of the bacterial physiological network. Physiological networking and signalling mechanisms increase (amplify) any cell disturbance, just as a cob-web increases small oscillations, and immediately provoke non-specific mechanisms of global adaptation. Phenotypic tolerance or formation of "persister cells" might be among this type of responses (see above), with mechanisms involving

the alarmone (p)ppGpp being involved in cell survival, and consequently in antibiotic resistance [40]. Other mechanisms might involve sigma factors, key-components of the translation cell machinery that are responsive to different types of stress [41, 42]. Sigma-S defective strains are more susceptible to antimicrobial agents [43]. Sigma-regulons are induced by beta-lactam agents, fosfomycin, teicoplanin, rifampicin, or polymyxins [44–46]. Probably heat-shock proteins also contribute to nonspecific antibiotic defence [47]. Of course that means that the excitement of global stress responses by factors other than antibiotics might non-specifically reduce the antibiotic potency. SOS adaptive response might also be unspecifically triggered by antibiotics. For instance, beta-lactam-mediated PBP-3 inhibition results in the induction of the SOS machinery in *E. coli* through the DpiBA two-component signal transduction system [48, 49]. Among the immediate consequences of such an early antibiotic sublethal effect is that bacteria might reduce their growth rate, eventually entering in some degree of phenotypic tolerance to drugs, and also that some other adaptive responses are triggered [49].

3.4 Genetic Variation: Mutation

3.4.1 Mutation Frequency and Mutation Rate

In the case of antibiotic resistance, the mutation "rate" is frequently and inappropriately defined as the in vitro frequency at which detectable mutants arise in a bacterial population in the presence of a given antibiotic concentration. Such a determination is widely considered an important task for the prognosis of the emergence of antibiotic-resistant bacteria. In the scientific jargon regarding antibiotics, a "mutation rate" is frequently presented in a characteristically naive way that can sometimes be understood as an intrinsic property of a new antimicrobial drug in its interaction with the target bacteria, with a "low mutation rate" that is considered an advantage over competitors. "This drug induces (?) a low mutation rate" is a familiar but completely mistaken expression. Note that in these types of tests we are recording the number of mutant cells and not the number of mutation events. In fact, we are recording only the selectively favourable mutations for the bacteria that lead to a visible antibiotic resistance phenotype, and therefore we are determining "mutation frequencies" and not "mutation rates". From the pioneering works of Luria and Delbrück, it became clear that evaluation of mutation rates is not easy. The methods for distinguishing the value of the observed frequency of mutants from the real mutation rate are not easy to apply, and fluctuation tests for analysis of the presence of populations of pre-existing mutants in the tested populations should be applied here. In the case of antibiotic resistance, the problem is complicated by the fact that the phenotype does not always reflect the same genotypes in all selected mutants, as mutations in different genes can produce similar antibiotic resistance phenotypes. For example, when a quinolone resistance mutation rate is determined, this rate is really the result of the combination of the mutation rates of the genes that encode the synthesis of GyrA, GyrB, ParA, ParC, and several different multidrug resistance (MDR) systems, and eventually other inactivating and target-protection mechanisms. In this respect, the calculated "phenotypic" mutation frequency is the result of several different "genotypic" mutation events.

The most important part of the adaptive possibilities of bacterial populations to environmental challenges, including adaptation to the anthropogenic antibiotic exposure, results from the huge quantity of bacterial individual cells. Simple calculations can provide an intuitive image of the mutation frequency in bacterial populations. *E. coli* genome has typically a size of 5,000,000 base pairs (5×10^6 bp), corresponding approximately to 5000 genes. The mutation rate of *E. coli* is 1×10^{-3} per genome (cell) per generation [50]. Divided by the number of genes, $0.001/5000 = 0.0000002 = 2 \times 10^{-7}$ p er gene and (cell) generation. Considering a cell density of 10^9 cells/ml in the colon, and a volume of 1000 ml in this part of the colonized intestine, we have 10^{12} *E. coli* cells in a single host (for instance, a particular patient) meaning that each day, supposing that *E. coli* divides only once/day in the colon, we have 200,000 mutations per gene/day for the entire *E. coli* population established in a single host. Of course resistance genes, or pre-resistance genes, will also evolve at this rate. Many *E. coli* clones are living in our intestine for years [51], so that the number of generations might be huge, and so the cumulative number of possible mutations offered to natural selection. How might bacteria tolerate such mutational load? Certainly due to purifying or stabilizing selection, that is, the alleles produced by most mutations are selectively removed if deleterious.

3.4.2 Hyper-mutation

The above calculations were based on huge bacterial populations in a shared environment (as *E. coli* in a "common" intestinal space in our example). However, many bacterial populations can be disaggregated, occupying small and eventually non-connected niches, with lower bacterial local densities in these compartments. Under immune response or antibiotic therapy, bacterial populations can also be reduced in size, and that applies in nature to all kinds of stressful conditions and bottlenecks. In environments where bacteria reach high population sizes, the normal mutation rates are more than enough to provide a sufficient wealth of mutational variation. However, when confined to low population sizes in compartmentalized habitats, variants with increased mutation rates (mutators) tend to be selected since they have an increased probability of forming beneficial mutations. Hyper-mutation is frequently due to the impairment of the

mismatch repair system, and more particularly involves alterations in *mutS* gene, but also in *mutL*, or *mutH*. Note that in an asexually reproducing organism, a mutator allele (for instance, the *mutS* allele that hyper-generates mutation) and the beneficial mutations are physically and genetically associated in the same chromosome. As a result the mutator allele will hitch-hike to increased frequency in the population together with the beneficial mutation.

One exemplary case is the selection of hyper-mutator populations in highly compartmentalized, chronic infections under frequent antibiotic exposure. This is the case of bronchopulmonar colonization in cystic fibrosis patients or those with bronchiectasis [52]. Determination of spontaneous mutation rates in *P. aeruginosa* isolates from cystic fibrosis patients revealed that 36 % of the patients were colonized by a hypermutable (mutator, mostly *mutS* deficient) strain (exceeding by 10–1000× the normal mutation frequency, 10^{-8}) that persisted for years in most patients. Mutator strains were not found in a control group of non-cystic fibrosis patients acutely infected with *P. aeruginosa*. This investigation also revealed a link between high mutation rates in vivo and high rates of antibiotic resistance [53]. An analogous rise in the proportion of hyper-mutable strains in cystic fibrosis patients has been documented for other organisms, including *Streptococcus*, *Haemophilus*, *Staphylococcus*, or *Stenotrophomonas*, and for analogous clinical conditions, as chronic obstructive pulmonary disease [54–56].

About 1 % of the *E. coli* strains have at least 100× the modal mutation frequency of 10^{-8} (strong mutators) and a very high proportion of strains, between 11 and 38 % in the different series, had frequencies exceeding by 4–40 times this modal value (weak mutators) [57] (Fig. 2.1). These proportions are obviously far higher than could be expected by random mutation of the genes that stringently maintain the normal mutation frequency. Moreover, increased mutation frequency may result in a loss of fitness for the bacterial population in the gut [58] as random deleterious mutations are much more frequent than the advantageous ones. Therefore the abundance of strains with increased frequency of mutation ought to be maintained by positive selection for the hyper-mutable organisms [59]. Without positive selection, the hypothesis is that these mutator populations would be extinct because of their unbearable mutational load (burden). However, we have shown in long-term evolution experiments that hyper-mutators might find mechanisms to reduce their rates of mutation, even if they cannot reacquire the repair function (for instance, the wild-type MutS gene) by horizontal gene transfer. These mechanisms involve protecting the cell against increased endogenous oxidative radicals involved in DNA damage, and thus in genome mutation [60].

The problem of combining the generation of variation required for adaptive needs and the required integrity of the bacterial functions might also be solved by strategies of low-level mutation, and "transient hyper-mutation". Possibly the fitness cost in terms of deleterious mutations is lower in a weak mutator and this allows their rising to higher frequencies in the population, and there might be a "reserve of low level mutators" in many bacterial populations, coexisting with the normo-mutable population. Indeed mutators are fixed in competition with non-mutators when they reach a frequency equal or higher than the product of their population size and mutation rate [61]. In populations of sufficient size, advantageous mutations tend to appear in weak mutators, and the selective process will therefore enrich low mutating organisms. The adaptive success of weak mutators may indeed prevent further fixation of strong mutators [61]. The "transient hypermutation" strategy will be treated in a paragraph below.

Striking differences have been found in the frequency of hyper-mutable *E. coli* strains depending on the origin; faecal samples of healthy volunteers, urinary tract infections, or bloodstream infections. *E. coli* strains from blood cultures are typically isolated from hospitalized patients and are therefore expected to have had a longer exposure to different hosts and antibiotic challenges. For instance, the frequency

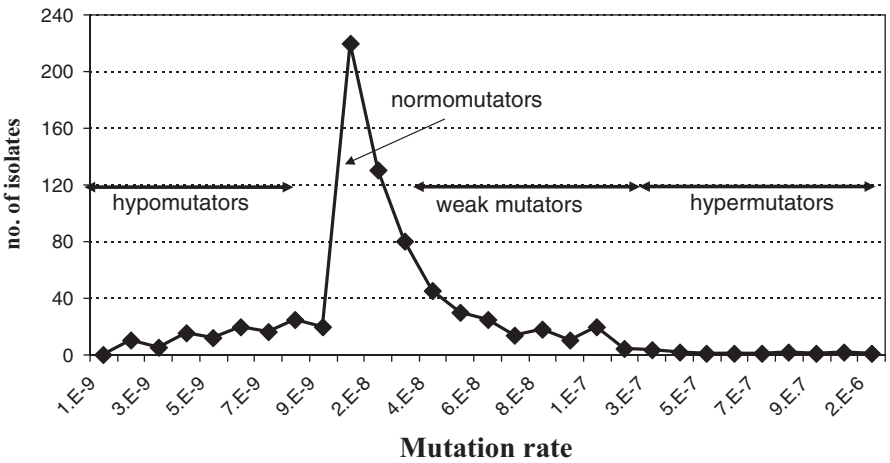

Fig. 2.1 Distribution of mutation frequencies for rifampicin-resistance in a large international series of *Escherichia coli* isolates recovered from patients and healthy volunteers. Hypermutators only account for 1 % of the strains, but weak-mutators are frequently found in clinical strains, but rare among healthy volunteers [37]

of hyper-mutable *E. coli* strains is higher among *E. coli* strains producing extended-spectrum beta-lactamases [62]. In general, adaptation to complex environments, including pathogenic ones, and the facilitation of between-hosts spread, leads to a certain microevolutionary "clonalization" (predominance of a particular clonal variant in a particular environment), which is facilitated by hypermutation [63]. In summary, mutation rates show a certain degree of polymorphism, and differences between isolates might reflect the degree of unexpected variation of the environment in which they are located [53, 64–67].

3.4.3 Antibiotics Inducing Mutations: Transient Mutation

A number of antibiotics induce adaptive responses to their own action, frequently—but not exclusively—by induction of the SOS repair system. SOS induction might be mediated by the SOS repair systems, not only those acting on DNA, but also on cell wall, as previously stated. One of the non-SOS effects (LexA/RecA independent)related to the PBP3-inhibition cell-wall damage response is the induction of *dinB* transcription, resulting in the synthesis of an error-prone DNA polymerase IV [68]. The consequence of this is an increase in the number of transcriptional mistakes, which might result in the emergence of adaptive mutations producing resistance to the challenging agents [67, 69]. Antibiotics that produce mistranslation, as aminoglycosides, induce translational stress-induced mutagenesis (non-inheritable!) [70]. Many antibiotics induce the SOS repair system, resulting in mutational increases, not only DNA-damaging agents, such as fluoroquinolones [71], but also beta-lactam agents [72]. The reason for mutational increase is the SOS-mediated induction of alternative error-prone DNA polymerases PolII, PolIV, and PolV.

3.5 Genetic Variation: Gene Recombination, Gene Amplification

Gene recombination might act as a restorative process which opposes gene mutation. Indeed a mutated gene, leading to a deleterious phenotype, might be replaced by homologous recombination with the wild gene if it is accessible in the same chromosome, or in other replicons of the same or different organism. For instance, if a mutated gene leading to antibiotic resistance is associated with a high biological cost in the absence of antibiotics, reducing fitness of the resistant organism, the mutated gene could be replaced by the wild-type gene, restoring both fitness and antibiotic susceptibility. This phenomenon might explain the partial penetration of some resistance traits in bacterial populations.

On the contrary, gene recombination might assure spread of mutations associated with antibiotic-resistance phenotypes. This might occur inside the same bacterial cell (intragenomic recombination) or between cells; in the last case, horizontal genetic transfer is required. Intragenomic recombination facilitates spread of homologous repeated genetic sequences. Gene conversion assures non-reciprocal transfer of information between homologous sequences inside the same genome. This might lead to minimizing the costs associated with the acquisition of a particular mutation (replacing the mutated sequence), or, on the contrary, to maximizing the benefits of mutations that confer a weak advantage when present as a single member (spreading copies of the mutated sequence) [73]. For instance, single-mutated rRNAs easily produce antibiotic resistance to aminoglycosides (and probably this is the case for other antibiotics) when the rest of the copies of rRNA sequences remain unchanged: the advantageous mutation spread by gene conversion [74].

Recombination in fact provides an extremely frequent mechanism for bacterial adaptation, being reversible in many cases. **Gene duplication-amplification** processes (either RecA-dependent or RecA-independent) are highly relevant in the adaptation to antibiotic exposure because they generate extensive and reversible genetic variation on which adaptive evolution can act [75–77].

For instance, sulfonamide, trimethoprim, or beta-lactams resistance (including resistance to beta-lactam plus beta-lactamase inhibitors) occur by increased gene dosage through amplification of antibiotic hydrolytic enzymes, target enzymes, or efflux pumps [78]. These cells now are now selectable by low antibiotic concentrations, increase in number and therefore also increase the probability for new adaptive mutations occurring in one of the amplified genes, eventually leading to higher levels of resistance. Once that occurs, low-level resistance by amplification-only is no longer efficiently selected. Moreover, gene amplification is inherently instable, and also might produce fitness costs, as each additional kilobase pairs of DNA reduces fitness by approximately 0.15% [79] so that the amplification will return to the original single gene-status. No signal will remain of this transient event in the genome sequence, and that is the reason why this evolutionary mechanism remains underdetected.

The possibility of gene recombination between bacterial organisms is highly dependent on the availability of horizontal gene-transfer mechanisms and the acceptance by the recipient cell of the foreign DNA. For instance, DNA uptake in *Neisseria meningitidis* or *Haemophilus influenzae* is highly sequence-specific. Transformation with *Streptococcus pneumoniae* DNA is exceptional outside this genus. In these very human-adapted organisms, intrageneric transfer facilitates the required variability in the surface proteins needed for colonization of mucosal surfaces in the human host, but the same strategy has been applied for optimizing mechanisms of antibiotic resistance. A variety of mosaic (hybrid)