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.

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

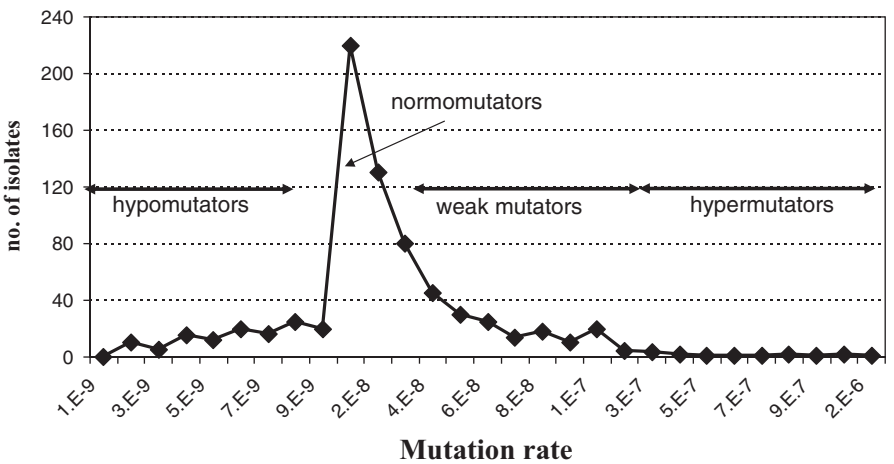

Fig. 2.1 Distribution of mutation frequencies for rifampicin-resistance in a large international series of *Escherichia coli* isolates recovered from patients and healthy volunteers. Hypermutators only account for 1 % of the strains, but weak-mutators are frequently found in clinical strains, but rare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

genes, encoding antibiotic-resistant variants of the target-proteins for beta-lactam antibiotics, have appeared in those organisms which are under antibiotic pressure. In aminoglycosides and in tetracyclines, mosaic hybrid genes are also frequent [80, 81].

3.6 Genetic Variation: Modularization

Modularization is a process by which variability is produced as a consequence of the building-up of different combinations among modular genetic elements, creating alternative genetic orders. Genomes of bacterial communities, species, and plasmids, and transposons, and integrons, frequently harbour or are constituted by modular genetic units. Genetic modules are any kind of repeated, conserved cohesive genetic entities that are loosely coupled [41, 82]. In fact there is a modular organization of nature, in which modules from different (non-genealogical) origins interact and integrate at different hierarchical levels [83]. Common or highly related genetic sequences (from small to very large ones) encoding resistance traits or associated with resistance genes have been found among different bacterial organisms, frequently belonging to different species and phylogenetic groups. The commonality of these sequences can be explained by a common phylogeny, by convergent evolution, or, probably more frequently, by lateral transmission of modular units, in a kind of reticulate evolutionary process. Incremental modularization, the addition of new "resistance" modules to a particular region might occur because there is a "module-recruiting" module (for instance, a recombinase), or by duplication of a pre-existing module, or by insertion of an incoming module. As the incoming modules or multi-modular structures frequently provide new interactive sequences, module accretion increases the local possibilities of recruitment of new modules. As this process of modularization occurs at particular genetic regions, these tend to become highly recombinogenic and module-promiscuous (high-plasticity zones). The cumulative collection of antibiotic resistance traits within particular multi-modular structures (integrons, transposons, plasmids) results from this type of nested evolution. The assemblage of modular components occurs by transposition, homologous recombination, and illegitimate recombinational events. Insertion sequences (ISs) are frequently involved in modularization. For instance, IS*26* mediates mobilization of bla_{SHV} genes encoding extended-spectrum beta-lactamases (ESBLs). The success of a plasmid containing one given $bla_{\text{CTX-M}}$ gene, as is the case of $bla_{\text{CTX-M-15}}$, also assures the spread of several IS*26* copies which might be involved in further modularization processes leading to multi-resistance [84].

The most beautiful example of the capturing efficiency of IS modules is the ability of the IS*Ecp1*B element to capture a wild beta-lactamase CTX-M-2 gene from the environmental organism *Kluyvera ascorbata* and mobilizing it into *E. coli*, that has now become resistant to third generation cephalosporins [85]. This recruiting module is involved in the expression and mobilization of many ESBLs [86]. Interestingly, the capturing ability of the IS*Ecp1*B module is dependent on a malfunctioning of this insertion sequence for excising itself in a precise way, and so integrating in the excising module sequences adjacent to the point of insertion. It has indeed been proposed that "imprecision" favours DNA arrangements and modularization. Another highly efficient IS module capturing and transposing not only ESBLs, but also metallo-beta-lactamases or co-trimoxazol, aminoglycoside, chloramphenicol and even fluoroquinolone resistance and even large chromosomal modules (genomic islands) are IS*CR*-type modules [87]. IS*CR*, IS with CR (common region), is a designation that implicitly reflects the modular structure of the module itself. A final example is IS*1999*, which when inserted upstream of a novel antibiotic resistance gene mediating very-broad spectrum beta-lactam resistance promotes its mobilization [88]. In principle, most modules involved in adaptive functions, including antibiotic resistance of every kind (from detoxifying enzymes to porin genes) might be recruited and translocated by IS modules. Other elements involved in module mobilization are DNA transposons and retrotransposons (that move by means of an RNA intermediate).

Modularization might act at the genome level as mutation acts at gene sequence level. Just as in the case of mutations, we should admit stochasticity as the major source of different modular combinations. We can expect that probably most of the combinations do not provide any fitness benefit, or might even reduce fitness of some module-associated functions. Nevertheless, some models suggest that even in the absence of any selective advantage, genotypic modularity might increase through the formation of new sub-functions under near-neutral processes [89]. Certainly it might be well conceived that some of these combinations could provide some direct adaptive benefits to the host cell, such as antibiotic resistance. Probably, successful combinations tend to perpetuate the connection among particular series of modules that act more and more now as a single complex module. For this reason there is a synthetic dimension of modularity, during which evolution tends to a number of genetic and biological orders, in a "doll-inside-doll" model. Note that modularity implies that bacterial entities are not formed or maintained as strict hierarchies, either from the top down (from ecosystem, communities, species, phylogenetic sub-specific groups, clones, genomes, long or short genetic sequences), or bottom-up (from short genetic sequences to ecosystem).

Indeed we know that not every bacterial phylogenetic group within a given bacterial species is represented in different ecosystems; no single clone is equally distributed among different hosts; every plasmid is not present at equal frequency among different bacterial species or sub-specific

groups. We also know that every type of mobile element is not equally distributed in any bacterial clone within a species, nor transposon is inserted with similar frequency in each type of plasmid, nor any kind of integron in any transposon, nor any antibiotic-resistance gene in any integron. These disequilibria are probably the result of cumulated selective events, exerted simultaneously at different hierarchical levels [90].

3.7 Horizontal Genetic Transfer and Bacterial Variation

The history of most commonly identified antibiotic resistance genes follows its original capture by different genetic units and further mobilization of novel "operational units" containing such antibiotic resistance genes into composite platforms (often comprising integrons, transposable elements, and/or insertion sequences) within plasmids that facilitate multiple DNA rearrangements among disparate genetic entities. Evolution based on gene recombination and modularization is greatly facilitated by horizontal (or lateral) genetic transfer. In particular, many drug resistance determinants spread between bacterial cells and species using plasmids, conjugative transposons, and probably phages. The evolution of resistance on these elements occurs in a modular fashion by sequential assemblage of resistance genes in specific sequences which are frequently mediated by specialized genetic elements such as integrons and transposable elements.

3.7.1 Plasmids and Drug Resistance Evolution

A plasmid is a double stranded, circular, or linear DNA molecule capable of autonomous replication. Plasmids frequently encode maintenance systems to assure copy-number and self-perpetuation in clonal bacterial populations. A plasmid may encode for a long-life cell-killing substance that is detoxified by a short-life plasmid product. If the plasmid is lost, the bacterial host is killed. To a certain extent, the same strategy has been applied to antibiotic (or heavy metals) resistance; only the clones harbouring plasmid-determined resistance will survive in an antibiotic-polluted environment. Therefore, plasmids use selective forces for their own maintenance and spread, and their spread in bacterial populations may be proportional to the intensity of these forces.

Facing in the 50s an increasingly selective antibiotic environment, historical (pre-antibiotic) plasmids immediately incorporated antibiotic resistance determinants. The study of pre-antibiotic collections of plasmids strongly suggests that the appearance of resistance genes in plasmids has only occurred during the last five decades. Indeed the diversity of the main plasmid families remains relatively limited, illustrating the success in continuous adaptation and spread of old plasmids thanks to antibiotic-mediated selection.

An example is the recent dissemination of old plasmids due to the incorporation to their genetic sequence of genes encoding for ESBLs. Promiscuous FII chimeric plasmids, widely spread among Enterobacteriaceae already before antibiotic discovery, are responsible for the current pandemic spread of $bla_{\text{CTX-M-15}}$, or bla_{TEM}, among other antibiotic resistance genes [91–94]. These plasmids harbour operational genetic platforms containing gene capture units as IS*ECp1*, IS*26,* or IS*CR*s, thus recruiting diverse antibiotic resistance genes [93, 95].

In many cases, the final success of resistant clones depends on the sequential acquisition of adaptive features unrelated to antibiotic resistance, facilitating in some of them to spread between hosts and/or environments (epidemicity) [96–100]. Moreover, the recent outburst of the OXA-48 enzyme, a widely distributed carbapenemase, has been related with the insertion of Tn1999 into the *tir* gene, encoding a transfer inhibition protein. This results in a high transfer frequency of plasmid harbouring $bla_{\text{OXA-48}}$ that might explain the successful dissemination of this enzyme [101]. All these observations indicate that the total plasmid frequency in bacterial populations might be increasing as a result not only of the more and more extensive anthropogenic release of selective agents, such as antimicrobial agents, but also to the advantage that they provide in circulating among microbiomes of multiple hosts, or in mediating resistance to other environmental organic chemicals or heavy metals [102]. This plasmid increase might have consequences on the full evolutionary machinery of bacterial populations, enlarging the number and variety of genetic interactions. In self-transmissible plasmids, there is always a possibility of entering (particularly under stress) into a new host resistant to the new drug, which may harbour another plasmid determining resistance to this drug. Plasmids from natural populations of *E. coli* frequently show a mosaic modular structure. Apparently plasmid–plasmid interaction and coevolution of recombinants (modular exchanges) depends on the possibility of plasmid coexistence. Plasmid promiscuity is limited by the phenomenon of plasmid incompatibility (two plasmids in the same cell might compete for the replication site, so that only one will be maintained), which depends on the amino acid sequence of the replication initiator proteins (RIP). However, a plasmid might enter into a cell where an incompatible plasmid is located, and still, before segregation, will have time to exchange modular traits with the resident one; on the other hand, many plasmids collect more than one Rep protein to be able to be replicated even if one of its Rep proteins is shared with the resident "incompatible" plasmid. No wonder that a multiple antibiotic environment has led the plasmid evolution towards the acquisition of multiple antibiotic resistance determinants in a single replicon unit, and even in the same gene cluster.

The possibility of a progressive increase in plasmid frequency and diversity (within classic plasmid backbones) in relation with an escalation of stressful and selective forces in nature, including antibiotic exposure, could be theoretically limited by plasmid incompatibility (inability of two related plasmids with common replication controls to be stably propagated in the same cell line), and progressive capture of plasmid genes by chromosomal sequences which make the cost of plasmid maintenance unnecessary. Recent advancements in the methods to determine plasmid relatedness, by restriction fragment pattern analysis, by classification into incompatibility groups (Inc), by PCR-based replicon (rep) typing (PBRT) [103], or more significantly by relaxase-typing by PLANC-Net methodology [104], or plasmid reconstruction in metagenomic analysis [105] have permitted the analysis of large series of resistance plasmids. These studies suggest that the limitation of plasmid incompatibility might be eventually surpassed by the evolution of multi-replicon plasmids or by plasmid co-integration.

An important point that is worth being investigated in more depth is the basis for specific stable maintenance of given plasmids in particular hosts. The development of solid systems for phylogenetic classification of sub-specific groups of bacteria are revealing that particular types of plasmids which eventually harbour particular types of resistance determinants are preferentially present in particular lineages. These bacterial lineages are acquiring the ever-lasting advantage of hosting evolutionary-active, plastic (modular) plasmids. The maintenance of a given type of plasmid in a given host depends on the "plasmid ecology" within the cell (host-plasmid mutual dependence, restriction-modification systems, presence of other plasmids), the reduction in the costs of maintenance, the rate of intra-populational transfer, and the frequency of selection for plasmid-encoded traits. The concept of specific stable maintenance means that, despite the potential transferability of plasmids to different hosts, some of them will be privileged in hosting particular plasmids, and these lineages or clones should have an increased evolvability in terms of developing antibiotic resistance.

But also mobile genetic elements might be shared by "genetic exchange communities", as "common adaptive goods" for ecologically integrated groups of (generally related) organisms [106, 107]. In such a way, antibiotic resistance tends to assure not only the survival of a particular lineage, but also of clouds of lineages, or even consortia of ecologically and functionally interconnected bacterial communities.

3.7.2 Transposable Elements

It is mainly transposable elements that have produced genetic transference of resistance in *S. aureus* and other gram-positive organisms. Class I transposons are able to mobilise themselves among different DNA sequences due to the presence of IS flanking their structure [108]. Different examples of Class I integrons are those involved in the transference of aminoglycoside resistance genes such as streptomycin, kanamycin or bleomycin (Tn5), chloranphenicol (Tn9) and tetracycline (Tn10). Tn4001, which is associated with IS256, is one of the most successfully disseminated transposons among gram-positive organisms. This element harboured the *aac6′-aph2″* gene which encodes a bifunctional enzyme able to inactivate most of the aminoglycoside antibiotics [109].

Class II transposons are widely disseminated among both gram-negative and gram-positive bacteria. They have a complex structure, which allows their mobilization from the bacterial chromosome to plasmids present in the bacteria. They have a genetic structure flanked by inverted repeat sequences which also include sequences with functional activity (transposase and resolvases) that facilitate their recombination and integration within the chromosome or a plasmid sequence. Some of these class II transposons contain resistance genes such as Tn*3* which harbour $bla_{\text{TEM-1}}$ gene or Tn*21* and their derivatives containing mercury or cadmium resistance genes, which may act as cofactors in the selection process [110, 111]. Another example of class II transposons are Tn*916*-Tn*1545* harbouring a tetracycline resistance gene in Enterobacteriaceae or Tn*1456* encoding glycopeptide resistance in enterococci. Moreover, some transposons are able to be transferred with a circular structure similar to that of plasmids (conjugative transposons). Some examples include tetracycline resistance (*tetM*) in *S. pneumoniae* or enterococci.

Transposons are important in the dissemination and maintenance of resistance genes and resistant bacteria. A transposon can be inserted inside another transposon and may contain more than one resistance determinant or even integron structure [109]. These latter elements are able to capture resistance genes (cassettes) due to the recognition of homologous sequences (integrase) and facilitate their expression [111, 112]. In general, bacteria harbouring integrons are more resistant to antimicrobials than those lacking these structures as an integron may present more than one resistance cassette. It is important to note that integrons can be mobilized by transposable elements which are also located in plasmids. This structure can be considered as an example of the "doll-inside-doll" model which undoubtedly gives advantages for the selection of resistant bacteria.

Most of the integrons have been described in organisms of high sanitary importance such as *Salmonella* Typhimurium, ESBL-producing *K. pneumoniae,* or *E. coli*. Within the integrons, class I integrons (according to the type of the integrase) have been successfully disseminated probably due to their integration in transposable elements and plasmids. The best example is that of integrons associated with the IS*CR1* structure (or ORF513) that are commonly associated with certain ESBL genes (*blaCTX-M*), carbapenemase genes, the *qnrA* gene which produces quinolone resistance, or ammonium quaternary compound resistance [84, 113].

3.7.3 Phages

The association of antibiotic resistance with bacterial phages has been overlooked for decades. We should remember that bacteriophages are probably the most abundant organism on Earth. Their ability to insert in bacterial genomes, to excise from them eventually carrying host DNA sequences, and to transfer to other bacterial cells, makes them potential vectors for disseminating antibiotic resistance. A number of examples of antibiotic resistance genes spreading by generalized or specialized phage transduction are available for *E. coli*, *P. aeruginosa, Staphylococcus epidermidis*, *S. aureus*, and *Actinobacillus. Burkholderia cepacia* transduce the resistance determinants to cotrimoxazol, trimethoprim and erythromycin to *Shigella flexneri*. A multiresistance gene cluster (*tetG, floR*, bla_{PSE1}) has been transduced from *Salmonella enterica* serovar Typhimurium DT104 to other serovars of *S. enterica*. A wide variety of β-lactamases (bla_{OXA-2}, bla_{PSE-1}, bla_{PSE-4}, or *bla*P) from *Proteus* have been found associated with bacteriophages isolated from sewage samples. The study of the genetic environment surrounding $bla_{CTX-M-10}$ β-lactamase gene has revealed the presence of upstream sequences with homology to conserved phage tail proteins [114]. It is not known whether these genes are part of a functional phage carrying $bla_{CTX-M-10}$ gene or only a reminiscent of an ancestral transduction event.

Abundant phage particles have been found in the supernatant of *Streptococcus pyogenes* harbouring the proton-dependent macrolide efflux system encoded by *mef(*A) gene, and these phage preparations have conferred macrolide resistance to a macrolide-susceptible strain [115]. High throughput sequencing has revealed phylogenetically diverse macrolide-resistant *S. pyogenes* strains carrying *mef(*A) inserted in different prophage or prophage-like elements, as Tn*1207.3,* alone or in combination with *tet(*O) gene. *Bacillus anthracis* carries a very diverse array of phages; among them are γ phages which contain a gene conferring resistance to fosfomycin. Bacteriophages isolated from food might in fact contribute to the propagation of antibiotic resistance [116].

3.8 Genetic Variation: Clonalization

Bacterial populations inside species are frequently subdivided in clones, particular lineages or units of descent that probably reflect different evolutionary histories. Multilocus sequence typing has pointed out that most isolates in a clonal population belong to one of a limited number of genotypic clusters (clonal complexes) that are thought to emerge from the rise in frequency and subsequent radial diversification of clonal founders [117, 118]. Rise in frequency is in most cases the consequence of selective events favouring the outburst of particular clones and clonal complexes in particular environmental circumstances. Each clone will correspond to a fitness peak, to an "ecotype" [119]. This means that the clonal structure of a bacterial population might reflect the changing variety of environments (including environmental gradients) to which the *ensemble* of the species is regularly exposed, and small changes among clones favours microevolution [117]. Therefore, we can conceive a bacterial species as a macro-structure composed of a number of clones and clonal complexes that might or might not be present in a particular location. In this sense, clones might behave as adaptive modules of a hierarchical superior entity, a "regional community structure", able to provide alternative stable states [120]. Mobile elements containing antibiotic resistance genes, such as plasmids, might circulate more effectively in such a genetically highly homogeneous multi-clonal structure, leading to typical complex endemic antibiotic-resistance situations [121] also termed resistance "allodemics" (see Sect. 4.3.1), and Fig. 2.2 [122–124].

3.9 Generation of Variation in Response to Antibiotic Stress

We have shown in Sect. 3.4.3 the influence of antibiotics on the mutation rate. Indeed that is a particular case of adaptive response to stress. Mutational events (base substitutions, frameshifts, excisions, insertions, transpositions) are increased by orders of magnitude under stress [125–127]. Probably, bacterial cells under extreme antibiotic-provoked stress (with membrane or cell wall damage, or compromised protein synthesis, or altered DNA supercoiling) could increase the rate of mutation, which would result in this type of adaptive response. Mutation rates can transiently increase depending on conditions of bacterial growth like starvation and environmental situations that cause bacterial stress, including induction of the SOS response. The SOS cascade can be induced by numerous antibiotics, presumably because these antibiotics cause the production of ssDNA [128]. DNA topoisomerase subunit A inhibitors, such as ciprofloxacin and other quinolones have a strong inducer SOS response [71, 129], however the subunit B inhibitors as novobiocin are not inducers [130]. On the other hand, antibiotics are also enhancing gene spread among bacterial populations: macrolides, tetracyclines, and beta-lactam agents facilitate intracellular and intercellular gene transfer. Conjugational transfer of the antibiotic-resistant transposon Tn*916* containing a tetracycline-resistance determinant, increases more than 1000-fold in the presence of tetracycline [131]. Most prophages are SOS-inducible so that SOS-inducing agents will dramatically increase the spread of prophages. This might significantly influence the spread of antibiotic-resistance genes [132], as it does for virulence factors. Indeed antibiotics might contribute to the spread of resistance genes modifying virulence and host-to-host frequency of transfer.

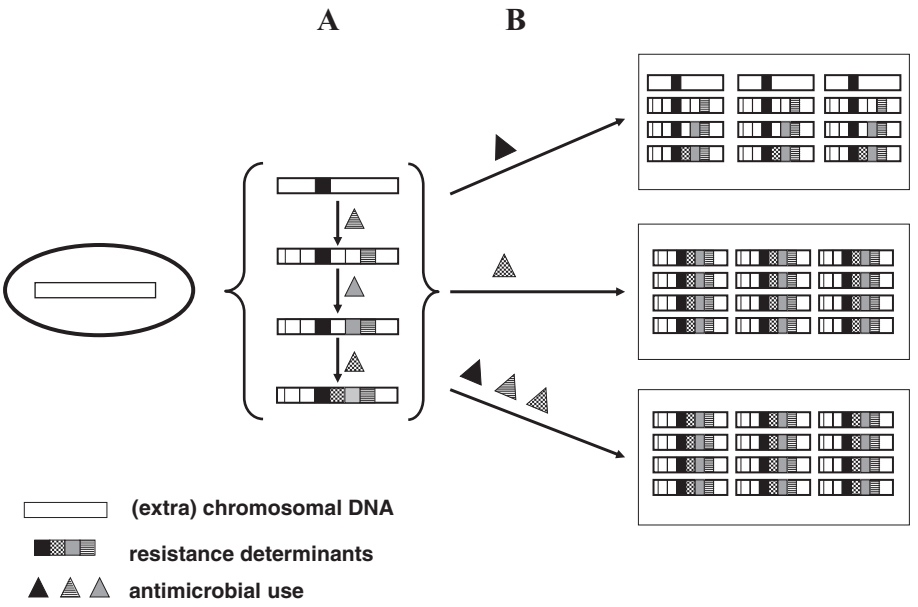

Fig. 2.2 Emergence of multi-resistance by sequential acquisition of antimicrobial resistance determinants (mutation or gene transfer) and selection of resistant bacteria under different antimicrobial selective pressures. (**a**) The sequential exposure to different antimicrobials may accumulate resistance determinants in bacteria. (**b**) The use of different antimicrobials may select resistant bacteria with different patterns of resistance determinants; note that eventually exposure to a single antibiotic produces the same selective effect for multidrug resistance that exposure to different drugs

For instance, the prophage-encoded shiga-toxin gene is SOS-induced and treatment of the hemolytic-uremic syndrome with SOS-inducers, such as fluoroquinolones, worsens the syndrome, amplifying the population of phages encoding shiga toxin [133]. Goerke et al. have demonstrated the increase of the expression of virulence factors and titres of particle phages in *Staphylococcus aureus* strains carrying ϕ13 lysogen, after being exposed to concentrations of ciprofloxacin near the threshold of growth inhibition [134, 135]. Other antibiotics such as trimethoprim have also been reported to cause phage induction [135]. In summary, antibiotic pressure in the environment may well contribute simultaneously to the increase in mutant-resistant phenotypes, to the selection of the fittest among them, and to the dispersal of resistance genes, which is expected to result in an acceleration in the rate of microbial evolution.

3.10 Phenotypic Variation and Genetic Variation: The Baldwin Effect

As stated in an earlier section (3.1) there is a certain degree of plasticity in bacterial cells and populations that are able to tolerate a determined concentration of antibiotics without requiring any inheritable genetic change. Regulatory factors influencing DNA supercoiling, catabolic repression or growth-phase specific regulators, translational modifications, and/or induction or stress responses might provide this flexibility. In a certain sense, the mechanisms of resistance that are induced by the presence of antibiotic agents also provide adaptive phenotypic variation, as is the case of AmpC related chromosomal beta-lactamases in *Enterobacter* or *P. aeruginosa* [136]. A classic important and still unanswered question in evolution is whether survival provided by phenotypic variation influences the emergence of specific inheritable genetic changes [137]. Apparently, phenotypic variation should limit the selective power of antibiotics for heritable changes, slowing evolution. Nevertheless, plasticity might help crossing adaptive valleys in a fitness landscape. For instance, antibiotic selection will favour the cells in the plastic population that are the most effective in resisting antibiotic action. Low-level antibiotic-resistance mutations arising in this population will probably be more effective than in the cells with lower expression of plasticity, and might be hooked by selection. Cells that are super-inducible for resistance might be prone to evolve to constitutive production of the mechanism. Indeed, a stress-inducible phenotype could be selectively enriched to the extent where it is stably (constitutively) expressed in the absence of stress [138].

4 Selection: The Mechanism of Evolution of Drug Resistance

The common wisdom supports that the emergence of drug resistance is a direct consequence of the selective events imposed by the use of antibiotics in clinical infections. That is probably true in terms of clinically relevant antibiotic resistance, involving a relatively high number of strains with high levels of resistance. In reality, the mere discovery of an antibiotic effect frequently reveals the presence of resistance to this antibiotic, and in many occasions the description of relevant mechanisms of resistance precedes the launching of the drug for clinical use (Table 2.3). Resistance is always there.

Table 2.3 Chronological introduction of different antimicrobial agents in therapeutics and emergence of resistance mechanisms

Antimicrobial agent	Discovery (introduction)	Resistance 1st reported	Mechanisms of resistance	Organisms
Penicillin G	1940 (1943)	1940	Penicillinase	*Staphylococcus aureus*
Streptomycin	1944 (1947)	1947	S12 ribosomal mutations	*M. tuberculosis*
Tetracycline	1948 (1952)	1952	Efflux	*Shigella dysenteriae*
Erythromycin	1952 (1955)	1956	23S rRNA methylation	*Staphylococcus aureus*
Vancomycin	1956 (1972)	1988	D-Ala-D-Ala replacement	*Enterococus faecalis*
		2004	D-Ala-D-Ala replacement	*Staphylococcus aureus*
Methicillin	1959(1961)	1961	MecA (PBP2a)	*Staphylococcus aureus*
Gentamicin	1963 (1967)	1969	Modifying enzymes	*Staphylococcus aureus*
Nalidixic acid	1962 (1964)	1966	Topoisomerase mutations	*Escherichia coli*
Cefotaxime	1975 (1981)	1981	AmpC ß-lactamases ESBLs	Enterobacteriaceae
		1983		Enterobacteriaceae
Imipenem	1976 (1987)	1986	Acquired carbapenemases	*Pseudomonas aeruginosa*
				Serratia marcescens
Linezolid	1979 (2000)	1999	23S RNA mutations	*Staphylococcus aureus*
				Enterococcus faecalis
Daptomycin	1980 (2004)	2005	Cell wall thickening	*Staphylococcus aureus*
				Enterococcus faecalis
Ceftaroline	2003[a] (2010)	2012	PBP modifications	*Staphylococcus aureus*

[a]Year of first publication

4.1 Selection by Low Antibiotic Concentrations

Antibiotic resistance is frequently recognized by clinicians as a therapeutic problem only after an extremely prolonged period of "subclinical resistance". During this cryptic period, a huge number of selective and evolutionary events take place among the originally susceptible bacterial populations challenged by continuous, intermittent or fluctuating antibiotic pressure, in the same or in different hosts. Bacterial spontaneous variability, perhaps increased after antibiotic-mediated mass extinction events, offers the selective process an important number of mutants, some of them exhibiting very low levels of antibiotic resistance. In most cases, these mutants remain indistinguishable from the fully "susceptible" strains applying the current standard susceptibility testing procedures that (implicitly) assume their selectability, considering that the peak antibiotic concentration in serum by far exceeds the concentration needed to inhibit the variant. Nevertheless, retrospective genetic and populational analysis of recently emerging resistant bacterial organisms, such as beta-lactam-resistant *S. pneumoniae* or *Enterobacteriaceae* harbouring ESBLs or carbapenemases, strongly suggests that low-level resistant variants have indeed been selected during treatments, and that they have evolved, after new cycles of mutation and selection, to high-level resistant organisms.

The discussions on the evolution of antibiotic resistance in microorganisms have been greatly dominated by some a priori beliefs. The first of them probably originated from human chemotherapy: to be considered "resistant" to an antibiotic, a given microorganism should express a relevant increase in the minimal inhibitory concentration (MIC) to this drug. In this view, "minor" increases are meaningless, since the patient can still be successfully treated with antibiotic concentrations exceeding this MIC value. A derivative belief is that: "only significant antibiotic concentrations apply in selection of resistance". Therefore, as antibiotics are mostly excreted in very small amounts by natural microorganisms in the environment, the origin of resistance as a result of these small selective forces (outside of the producing organism) tends to be disregarded. A third belief, closely related to the first, is that "resistance genes" are only those related to "significant" high-level resistance. Under natural circumstances, the preservation of susceptible bacteria may depend on the fact that the selective effect could be preferentially exerted in a given spatial compartment, in a "small niche" according to Smith and Hoekstra [139]. We propose that this compartment, responsible for this type of "confined selection", could be considered as the space or niche in which a precise concentration of antibiotic provides a punctuate selection of a particular resistant bacterial variant. The antibiotic concentration exerting such an effect is here designated as the "selective antibiotic concentration".

4.2 Concentration-Specific Selection: The Selective Window

Any antibiotic concentration can potentially select a resistant variant if it is able to inhibit growth of the susceptible population but not that of the variant harbouring the resistance mechanism. In other words, a selective antibiotic concentration is

that which exceeds the minimal inhibitory concentration (under the local conditions) of the most susceptible population, but not that of the variant population (even if it is very close). If MICs of both susceptible and variant populations are surpassed, then no selection of the variant is expected to occur, and the same applies when the antibiotic concentration is below the local MICs of both populations. Therefore, the selection of a particular variant may happen only in a very narrow range of drug concentrations [140].

Among the more efficient new TEM-beta-lactamase variants that have evolved to hydrolyze cefotaxime are those which differ from the earlier molecules by several amino acids. Assuming the known mutation rates in *E. coli* (see above) it is unlikely that two or more point mutations would appear simultaneously in a beta-lactamase gene. Therefore, if the TEM-1 beta-lactamase is the ancestor of these multiple multiplied variants, it is most likely that the variants arose by a process of sequential point mutation and selection of singly mutated intermediates. For such a scenario to be plausible, each mutation would need to confer a selective advantage over the ancestral strain. In many cases, strains with monomutated TEM-1 enzymes (such as TEM-12, resulting from a single substitution of arginine for serine at position 164) exhibit only a very small increase in resistance to cefotaxime. Typically, TEM-1-producing *E. coli* is inhibited by 0.008 μg/ml, and TEM-12-producing *E. coli* is inhibited by 0.015 μg/ml. Both in vitro and in vivo experiments have demonstrated that despite such a small phenotypic difference, TEM-12-containing strains are efficiently selected by cefotaxime exposure, thereby providing the genetic background for double-mutated, more efficient enzymes for example TEM-10 [141]. Such selection only occurs in particular antibiotic concentrations that define a "selective window for selection" [6].

4.3 Antibiotic Gradients in Antibiotic Selection

Sublethal antibiotic concentrations are able to efficiently select for antibiotic resistance [142, 143]. At any dosage, antibiotics used in chemotherapy create a high diversity of concentration gradients, which inevitably include sublethal (but selective) antibiotic concentrations. These gradients are due to pharmacokinetic factors, such as the different diffusion rates into various tissues, or variation in the elimination rate from different body compartments. The direct effect of microbes of the normal or pathogenic flora, that possess antibiotic-inactivating enzymes, also contributes to the gradient formation. Bacterial populations in the human body probably face a wide range of antibiotic concentrations after each administration of the drug. Since the spontaneous genetic variability of microbial populations also provides a wide range of potentially selectable variant subpopulations, it is appropriate to determine which antibiotic concentration is able to select one or other of these particular subpopulations.

Theoretically, each particular variant population showing a definite MIC will have the possibility of being selectively enriched by a particular antibiotic concentration. This conclusion appears obvious. Surprisingly, the theoretical and practical consequences of such a conclusion remain to be explored in the aim of a better understanding of the evolution of antibiotic-resistant bacterial populations. Bacterial populations show impressive natural genetic polymorphism. For many antibiotics, spontaneous gene variation frequently results in a multiplicity of low-level mechanisms of resistance and the emergence of more specific high-level mechanisms are less frequent (except for a limited number of antibiotics, or by uptake of exogenous highly specialized genes). In the real world, antibiotic concentrations challenging bacteria are mostly located in the low-level margin; those populations showing small increases in MIC would be expected to be preferentially selected by these antibiotics. We emphasize once more the importance of the selection of low-level resistant bacterial mutants to explain the spread of high-level resistance. First of all, several consecutive rounds of selection at the selective antibiotic concentration will produce a progressive enrichment of the low-level variant, and this occurs during most multi-dose treatments. Once a critical number is reached, new variants may arise which can then be selected in the following selective antibiotic concentration, so increasing the antibiotic resistance level. On the other hand, low-level resistant variants can arrive at a position permitting the incorporation of foreign resistance genes in an antibiotic-rich medium. In conclusion, these studies of population selective amplification suggest that at the different points of a concentration gradient, selective forces may be acting with different selective specificity. To a certain extent, the continuous variation of antibiotic concentrations may resemble a tuning device which selects a determined radio frequency emission. Under or over such a frequency (the antibiotic selective concentration), the emission (the particular variant) is lost (selection does not take place). The saddle between the concentrations inhibiting the susceptible and resistant populations is the frequency signal recognized by the selective antibiotic concentration.

A more practical conclusion has been developed in this field when Drlica and collaborators proposed to use antibiotics at dosages that should surpass the "mutant prevention concentration" to avoid the selection of resistance mutants [144].

4.4 Fluctuating Antibiotic Environments

Fluctuating antibiotic environments may facilitate the possibility of evolution of a resistant organism towards higher adaptive peaks than fixed environments. Despite the large

number of in vitro mutations that increase resistance to extended-spectrum cephalosporins in TEM-type beta-lactamases, only a small number occur in naturally occurring enzymes. In nature, and particularly in the hospital setting bacteria that contain beta-lactamases encounter simultaneous or consecutive selective pressure with different beta-lactam molecules. All variants obtained by submitting an *E. coli* strain that contains a $bla_{\text{TEM-1}}$ gene to fluctuating in vitro challenge with both ceftazidime and amoxicillin contain only mutations previously detected in naturally occurring beta-lactamases. Nevertheless, some variants obtained by ceftazidime challenge alone contained mutations never detected in naturally occurring TEM beta-lactamases. A number of modulating mutations might arise that are neutral by themselves but in addition to others might equilibrate the antibiotic substrate preference in fluctuating antibiotic environments [141]. Indeed it can be suggested that extended-spectrum TEM variants in hospital isolates result from fluctuating selective pressure with several beta-lactams rather than selection with a single antibiotic.

4.5 Selection Towards Multi-Resistance: Genetic Capitalism

The concept of genetic capitalism has been applied to MDR pathogens [90]. It refers to further adaptive possibilities of organisms to accumulate resistance mechanisms, either via mutational or gene acquisition events. This reflects a kind of genetic capitalism—the rich tend to become richer. In the last years different examples illustrate this concept such as methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, ESBL-producing Enterobacteriaceae, or carbapenemase-producing Enterobacteriaceae. Genetic capitalism has determined not only the increase in prevalence of MDR resistance pathogens but also the spread and maintenance of resistance genes among clinical isolates, those belonging to the microbiota and in the environment [145]. Obviously, in environments where exposure to different selective agents (antimicrobial drugs) is frequent, the organism harbouring more resistant traits should have higher possibilities of being selected (multi-lateral selection), and a single antibiotic might select multi-resistant strains. This process is illustrated in Fig. 2.3. Moreover, the acquisition of resistance genes, or even virulence traits, may increase clonal fitness and may facilitate the uptake of more and more adaptive advantages. Examples of dispersion of specific genes among bacterial isolates from different compartments are those conferring resistance to tetracyclines (*tet*), macrolides (*erm*), beta-lactamases (*bla*), aminoglycosides (*aac, aad, aph*), sulfonamides (*sul*), trimethoprim (*dfr*), and more recently colistin (*mcr*). In certain cases, the persistence of resistance genes such as those affecting sulfonamides and streptomycin cannot be explained by the current antibiotic selection pressure, as these antibiotics are scarcely used. However, the concomitant presence of other resistance genes may drive this selection process and explains this paradox. Moreover, the genetic support of resistance genes, including integrons, transposons or plasmids, also facilitates their persistence without selective force [146].

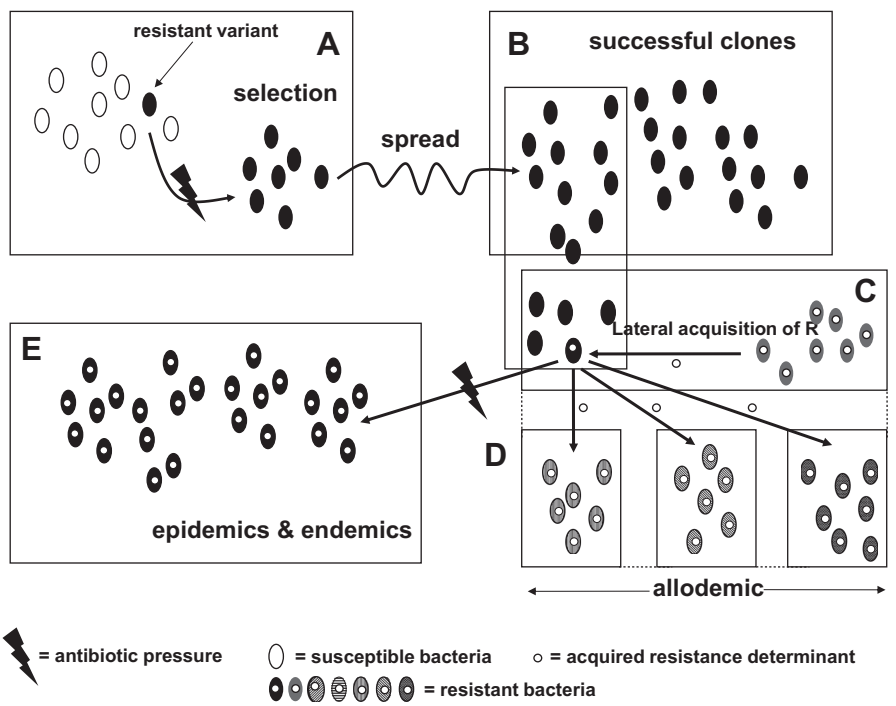

Fig. 2.3 Epidemiological scenarios for the selection and spread of antimicrobial-resistant bacteria: (**a**) the use of an antimicrobial agent may select resistant bacterial variants within a susceptible population; (**b**) selection might contribute to the dominance (success) of the resistant clones, favouring spread in different compartments; (**c**) because of the dominance, successful spreading clones are prone to contact with resistant organisms and to acquire resistance genes by lateral transfer processes; (**d**) at their turn, these resistant clones might act as donors of resistance to other clones depicting an allodemic (or polyclonal) resistance situation); (**e**) resistant clones with acquired resistance genes may become dominant in particular environments depicting epidemic or endemic situations

5 Evolution of Drug-Resistance: Future Prospects

5.1 Units of Variation and Units of Selection

What is selected when we speak about selection of antibiotic resistance? Evolution acts on variation of individual entities. Of course, an individual is not only a single cell, individual animal or plant. In general, an individual can be defined as any simple or complex structure with the potential to maintain, replicate, or reconstruct its self-identity, and also able to escape or at least postpone death, a destructuring or disordering process. Organisms are units of selection, evolutionary units, in a sense "evolutionary individuals", defined as any entity that, independently from the number of elements that enters into its composition or from its hierarchical level of complexity, is selected and evolves as a unit [147]. Because interactions lead to order, individuals should interact with one another. With this perspective, we imagine different kinds of individuals, including "primary order", or elementary individuals, but also secondary, tertiary, and still-higher orders, in which those simpler groupings form more complex assemblies. At any level of the hierarchy variation might occur, and, in a sense the individuals are also units of variation. The modern hierarchical theory of evolution suggests that all types of individuals, at several different levels of integration, independent objects of selective forces, offering a new perspective, one that may be considered as ultra- or hyper-Darwinism. In classic Darwinism, the ordering finger of evolution operates within the selfish organism and, in the later Dawkinian sense, the selfish gene. Ultra-Darwinism serves as a reminder that evolution may occur not only at the level of individual organisms and species, as conceived by Darwin it, but also at the sub- and supraorganismal levels.

Suborganismal evolution may involve molecules such as peptides and proteins. Thus, relatively simple forces, such as chemical stability in a certain environment or modular structures within a particular protein conformation, may exert selective pressures within the "protein universe". Suborganismal evolution may also involve genes; operons; stable chromosomal fragments; mobile genetic elements such as plasmids, transposons, integrons, and insertion sequences; and "nuons". This term, coined in 1992 by Brosius and Gould [148], encompasses any nucleic acids that could act as an elementary unit of selection. Thus, nuons might include genes, gene fusions, gene modules encoding protein catalytic domains, intergenic regions, introns, exons, promoters, enhancers, slippage regions, terminators, pseudogenes, microsatellites, and long or short interspersed elements. Organismal evolution is exerted on units of selection that are typically microbial clones or cell lineages with particular genomic contents, including also demes or local populations. Supra-organismal evolution is exerted on microbial species, with species considered here as a biological individual with a birth, a transformation and possible death; on clades which are monophyletic groups of species; on communities of microbial species, which include microbiomes, possessing metagenomes; and also on stable associations of microbiomes with particular hosts or host communities (metabiota). We frequently use the term "system" to describe the structure of individuals of higher complexity.

Because of that, the analysis of antibiotic resistance requires the study of the multi-level population biology of antibiotic resistance. Antibiotics have natural functions, mostly involving cell-to-cell signalling networks. The anthropogenic production of antibiotics, and its release in the microbiosphere results in a disturbance of these networks, antibiotic resistance tending to preserve its integrity. The cost of such adaptation is the emergence and dissemination of antibiotic resistance genes, and of all genetic and cellular vehicles in which these genes are located. Selection of the combinations of the different evolutionary units (genes, integrons, transposons, plasmids, cells, communities and microbiomes, hosts) is highly asymmetrical. Each unit of selection is a self-interested entity, exploiting the higher hierarchical unit for its own benefit, but in doing so the higher hierarchical unit might acquire critical traits for its spread because of the exploitation of the lower hierarchical unit. This interactive trade-off shapes the population biology of antibiotic resistance, a composed-complex array of the independent "population biologies". Antibiotics modify the abundance and the interactive field of each of these units. Antibiotics increase the number and evolvability of "clinical" antibiotic resistance genes, but probably also many other genes with different primary functions but with a resistance phenotype present in the environmental resistome. Antibiotics influence the abundance, modularity, and spread of integrons, transposons, and plasmids, mostly acting on structures present before the antibiotic era. Antibiotics enrich particular bacterial lineages and clones and contribute to local clonalization processes. Antibiotics amplify particular genetic exchange communities sharing antibiotic resistance genes and platforms within microbiomes. In particular human or animal hosts, the microbiomic composition might facilitate the interactions between evolutionary units involved in antibiotic resistance. The understanding of antibiotic resistance implies expanding our knowledge on multi-level population biology of bacteria [149].

5.2 The Limits of Drug-Resistance Evolution

5.2.1 Saturation Constraints, Short-Sighted Evolution

There are potential bottle necks for the evolution of antimicrobial resistance. For instance, genetic variation inside the modified target, determining more and more effective antibiotic resistance levels, may arrive to exhaustion. As the efficiency

of the mechanism of resistance improves incrementally, the selective advantage of each increment will diminish, until a saturation point is reached at which increments in functional efficiency result in negligible improvements in fitness [150]. Typically this may occur in enzyme kinetics (for instance, hydrolyzing ability of a beta-lactamase for a given beta-lactam antibiotic). When this stage is reached, random changes in amino acid sequence are more often expected to impair enzyme performance than improve it. In the case that the modified antibiotic target retains some vital functions in the bacterial cell, the mutational modifications required to reach very high-level antibiotic resistance may reach a lethal situation. This can be considered as a case of "short-sighted evolution".

5.2.2 Minimizing the Costs of Evolvability

In a well-adapted organism, any change including acquisition of drug-resistance, has a biological risk. Hence bacterial organisms have developed mechanisms to reduce variation to the lower possible level compatible with evolvability, evolutionary innovation, and ability to adapt. The most obvious way to reduce the necessary costs associated with variation is by reducing genetic variation itself, even at the expense of decreasing variability. The most basic mechanism reducing genetic variation is the degeneracy of the genetic code as a number of nucleotide changes are not reflected in changes in amino acid sequence (synonymous nucleotide substitutions). Variation is also reduced by assuring a high-fidelity transcriptional process during DNA replication, or by using highly effective mechanisms of repair of transcriptional mistakes, including increased homologous recombination or daughter strand gap repair. Interestingly, a number of bacteria might have evolved effective mechanisms to reduce the mutation frequency below the average (hypomutation). Mechanisms for stress reduction should also reduce evolvability; indeed the full adaptation of an organism to a very specific niche reduces stress, but stress is maximized when this well-adapted strain is obliged to leave its normal environment. A number of antibiotic resistance mechanisms involved in detoxification of a drug or its expulsion decrease antibiotic-mediated stress and probably reduce variation and evolvability [151].

As stated above, the biological risks associated with the acquisition of drug-resistance might be diminished by management of sequences determining such resistance in modules (relatively "external" to the basic cell machinery) and particularly modules contained in module-carrying elements (as plasmids).

5.2.3 Cost of Antibiotic Resistance

As said before, gene mutants that have been selected for novel resistance phenotypes may have maladaptive pleiotropic effects [152]. This means that acquisition of resistance may de-adapt the resistant organism to its environment thus reducing its competitiveness. Under antibiotic pressure, the competitor organisms may be incapable of taking advantage of this, and therefore the resistant bacteria genotypes have a chance to compensate maladaptation by selection of modifiers [153, 154]. This process of adaptation to its own resistance determinants may completely eliminate the biological cost of resistance. The costs associated with the acquisition of non-advantageous changes might be compensated by the acquisition of new changes. Intragenic or extragenic changes (including, for instance, restorative mutations, gene silencing, or excision) might compensate the cost in a particular environment, but this compensation might even increase the cost in other circumstances. Gene duplication might compensate for decreases in function of a mutated gene and this compensatory effect alone might have important evolutionary consequences. Interestingly, compensatory changes in the bacterial genome may be fixed for reasons other than antibiotic resistance, thus perpetuating the resistance characters in particular genotypes, even in the absence of antibiotic selection. Indeed chromosomal compensatory mutations may eventually increase the bacterial fitness, even if the antibiotic-resistant determinant is lost. At the same time, these organisms may be in the optimal situation of being able "without cost" to lose the mechanism if necessary. Frequently, resistant genes are located in large plasmids, but plasmid carriage usually reduces the competitive fitness of bacteria in the absence of selection for plasmid-encoded functions. It could be expected that plasmid-mediated antibiotic resistance may not be able to persist in bacterial populations in the case of discontinuation of antibiotic use. Interestingly, the cost of plasmid carriage may be compensated in some cases by the mechanisms of resistance encoded, even in the absence of selection. For instance, a tetracycline-efflux pump (determining resistance to this antibiotic) may be used for exporting toxic metabolites from the cell [154]. The in-practice non-functional bleomycin-resistance gene in plasmids harbouring the transposon Tn*5* may confer improved survival and growth advantage [155].

5.3 Epidemiology and Evolution of Antibiotic Resistance

Bacterial selection may result from the acquisition of resistance to environmental changes that are deleterious for competing populations as happens after exposure to antibiotics. Apparently, resistance does not add new capabilities to the survivor: it just compensates (equilibrates) the reduction in reproductive output imposed by the antibiotic. Consequently, immediate intuition associates selection of antibiotic-resistant microbes with the classic expression "*survival* of the fittest". Note that resistant organisms are only "the fittest" in the presence of antibiotics. Certainly natural selection also acts on positive differences when the acquisition of

a novel trait is able to increase the ability of the bacterial organism to exploit a given environment thus provoking a selective difference with the competitors. It is frequently unrecognized that antibiotic resistance provides this type of selective advantage, which is not only a compensation for a loss but *at the same time* is also the gain of a new possibility of habitat exploitation. Frequently, antibiotic-producing microorganisms simultaneously produce antibiotic-resistance mechanisms [31, 156]. It may be that the objective (benefit) of antibiotic production is to obtain an *exclusive* environment where only the producer is able to survive, because of resistance. As a consequence, all the resources of the environment can be exploited exclusively by the producing strain. In other words, in the presence of the antibiotic, antibiotic resistance is a colonization factor to gain *exclusivity* for resources. Etymologically, exclusive means "closed for the others". It may be well conceived that in a world in which antibiotics have become frequent components from microbial environments (in particular in humans and animals), the acquisition of antibiotic resistance is evolving not only a protective mechanism but also a factor assuring *exclusivity* for the resistant populations in antibiotic-containing areas. The increase in the absolute number of antibiotic-resistant organisms is the proof of the benefits of this strategy.

5.3.1 Resistance, Epidemics, Endemics, and Allodemics

Antibiotic resistance is expected to have a minor biological or clinical effect in the absence of effective spread of resistant organisms. As stated in the last paragraph antibiotic resistance might help a given organism to spread, particularly in environments assuring frequent exposure to these drugs. Eventually hyper-mutable organisms might be better suited for host colonization, host-to-host transmission, survival in inert environments, and also for developing antibiotic resistance, either by mutation or by homeologous recombination with exogenous genes. On the other hand, pathogenic and epidemigenic organisms are probably more frequently exposed to antibiotic therapy. Therefore, a certain convergence between virulence, epidemigenicity and resistance could be expected to occur [65]. Interestingly, antibiotic-resistant clones frequently coincide with "successful clones" well adapted for colonization or spread *before* acquiring antibiotic resistance. This convergent process of selection, leading to the dissemination of antibiotic resistance determinants in different bacterial populations is illustrated in Fig. 2.3. Examples of this can be found in beta-lactam-resistant *S. pneumoniae, E. faecalis* and *S. aureus* or in glycopeptide-resistant *E. faecium* [157–161].

However, consistent with the concept of the multiplicity of units of selection stated before (paragraph 5.1.), a particular epidemigenic "resistant clone" does not constitute the only selectable unit of antibiotic resistance. The wide application of molecular techniques, such as restriction pulsed field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) to the definition of bacterial clones have offered a totally new view of several 'epidemic' phenomena. A surprising diversity of clones was found when the clones responsible for the progressive and steep increase of enterobacterial strains harbouring ESBLs in a single hospital were studied. For instance, *K. pneumoniae* strains harbouring $bla_{\text{CTX-M-10}}$ or $bla_{\text{OXA-48}}$ belonged to more than thirteen different clones! Therefore the case was "epidemics of $bla_{\text{CTX-M-10}}$ or $bla_{\text{OXA-48}}$ resistance" and but not "epidemics" in the classic sense. The term "**allodemics**' (from Greek *allos*, other, different; and *demos*, people), in the sense of "something is being produced in the community by different causal agents" has been proposed to describe this pattern (Fig. 2.3) [122]. Note that the infection (or in our case the frequency of antibiotic resistance) may cluster but not necessarily its causative organism. In other words, the phenotype may cluster, but not the genotype. Indeed the concept of allodemics emphasizes the importance of the asymmetry between phenotype and genotype in natural selection. Its practical consequences are quite obvious. In documented allodemic situations, interventions should be focused more to the environmental causes of the problem than to the classical approaches including clone-directed measures to limit host-to-host spread, or search-and-destroy strategies. For instance, in our particular case, a reduction in the intensity of use of antibiotics potentially able to *select for* ESBLs or carbapenemases could be an appropriate environmental intervention for controlling allodemic situations.

5.3.2 Resistance as a Colonization Factor

In the absence of antibiotics, resistance does not generally add new basic capabilities to the physiology of the bacterial cell and often produces reduction in fitness. In other words, resistance does not "improve" the cell machinery but only just compensates (equilibrates) the reduction in reproductive output imposed by the antibiotic. From this point of view, can antibiotic resistance be considered a factor triggering important changes in long-term bacterial evolution?

Certainly, natural selection also acts on positive differences when the acquisition of a novel trait is able to increase the ability of the bacterial organism to exploit a given environment thus provoking a selective difference with the competitors. It is often unrecognized that antibiotic resistance provides this type of selective advantage and being not only a compensation for a loss, but *at the same time* the gain of a new possibility of habitat exploitation. Antibiotic-producing microorganisms produce antibiotic-resistance mechanisms simultaneously [31, 156]. When this occurs it may be that the biological benefit of antibiotic production is to obtain an exclusive environment, in which only the producer is able to survive because of resistance. The same might be true if a bacterial organism resistant to antibiotic A were able to

induce antibiotic A production in another antibiotic-producing organism such as another bacteria, fungus, plant or animal. Antibiotic release will eliminate competitors. In a certain sense, antibiotic-resistant bacteria have taken ecological advantage of human production and release of a number of antibiotics. The increase in the absolute number of antibiotic-resistant organisms is the proof of the benefits of such an evolutionary trend.

5.3.3 Biogeography and Local Biology of Antibiotic Resistance

Biogeography of resistance is the study of the distribution of diversity of resistance over space and time [162]. In the words of Brendan Bohannan, "space is the next frontier in biology". The world is a spatially structured place, with localized dispersal, localized interactions and localized selective events. In environments under high intensity of selective forces (for instance, in the hospital, because of pathogenesis, host-to-host spread, and local usage of antiseptics and antimicrobial agents), the local tool-kit of evolutionary active elements should be very large. Locally successful sub-specific groups, clones, plasmids, transposons, integrons or antibiotic resistance genes (see Sect. 5.1 about individuals and units of selection) will be cumulatively selected, and possibilities of interaction (accessibility-connectivity) will necessarily increase. Consequently in these environments we can expect acceleration in the evolution (construction-selection) of complex structures eventually involved in antibiotic resistance. Organisms that are ecologically and/or phylogenetically distant, present in a low density or submitted to environmental isolation might have reduced possibilities for genetic exchange and evolvability. The term "exchange community" has been proposed to identify the biological systems able to exchange genes [163]. It is possible that genetic exchange might occasionally occur among organisms sharing similar lifestyles across a wide phylogenetic range, as such "ecologically close" ensembles of organisms tend to conserve equivalent regulatory networks [164]. The presence of the same antibiotic resistance genes in ecologically connected bacterial genera indicates a complex history of genetic interactions in which antibiotic resistance genes have parasitized the natural circuits of adaptive gene flow. Note that "genetic exchange communities" are necessarily local ones [165]. Different environments with different cumulative histories of antibiotic use and local epidemics/endemics may harbour different ensembles of evolutionary pieces. Therefore the emergence and development of new antibiotic-resistance patterns is probably of biogeographical dimension [166]. Of course "global spreading clones" disseminate a number of the genetic elements involved in antibiotic resistance but once in touch with local biological ensembles, a local phylogeographic diversification tends to take place [167].

5.3.4 Antibiotics as Ecosystem-Damaging Agents: The Role of Resistance

Simply put, antibiotic agents are chaos-promoting factors for microbial ecosystems because these agents provoke functional disorders and death in many kinds of bacteria. The use (particularly the abuse) of such agents leads to collapse in the diversity of these microorganisms along with entire ranges of individuals. It can be stated that Nature will be always able to recover some degree of biological equilibrium. We should be aware that the extensive use and release of drugs may be provoking the emergence of new biological orders. It is difficult to predict whether these new orders will be better for the whole system or will lead to new adaptive difficulties. The short-term relief that we derive from using antibiotics may be followed by longer term difficulties that are the hallmark of any evolutionary trend.

Supracritical release of antimicrobial agents should disturb microbial populations, affecting many different types of individuals (units of selection) within those populations. Among individuals at the supracellular level, for instance, within intestinal bacterial communities or the soil microbiota at a particular site, the functional loss of bacteria within a particular system can be repaired by residual "redundant" populations that survive such a challenge, by degenerate populations of other bacteria fulfilling a similar function, by imported populations migrating from a connected system or eventually by the emergence of novel variant organisms. At the level of the individual organism—for instance, a single bacterial cell—redundant or degenerate genes can repair or otherwise overcome the damage that follows an antibiotic challenge. This reordering may depend on replacing those functions that the antibiotic inhibited, by importing foreign genes that can deactivate the antibiotic or by mutation- or recombination-dependent innovation that leads to antibiotic resistance. Because of the hypothesis of multiple-units of selection affected by antibiotics, these drugs might have a second-order evolutionary impact on suborganismal individuals—for instance, on plasmids, integrons, operons, genes, insertion sequences, and proteins. Critically, antibiotics or any other agent or circumstance promoting disorder may expand across the whole hierarchy of evolutionary individuals. For instance, local disordering events may select different types of bacterial clones in a particular environment, such as that within a specific hospital. Genes or proteins carried by these clones may be enriched. The amplifying selective process increases the possibilities of interaction among certain clones, genetic elements, and other molecules. The best combinations for local survival increase in number which facilitates further adaptive possibilities and reflects a kind of genetic capitalism—the rich tend to become richer. From this perspective, antibiotic resistance might constitute an ecological risk and at the same time—deactivating the effect of antimicrobial drugs—a factor of ecological protection.

5.3.5 Evolutionary Ecology and Spread of Antibiotic Resistance: The Four P's

All evolutionary ecology of antibiotic resistance, involving its spread and diversification can be summarized in the consideration of the four P's: (1) **P**enetration in microbial ecosystems, including microbiotas, of highly effective pathogenic clones and plasmids, (2) **P**romiscuity of genetic traits involved in antibiotic resistance by lateral gene transfer, (3) **P**lasticity of genetic vehicles and platforms, taking advantage of highly recombinogenic sequences, and (4) **P**ersistence and maintenance of different pathogenic multidrug-resistant high-risk clones and globally distributed plasmids [168]. These four P's determine the **three main processes** shaping the natural history of antibiotic resistance, involving the emergence, invasion, and occupation by antibiotic-resistant genes of significant environments for human health. The process of emergence in complex bacterial populations is a high-frequency, continuous swarming of ephemeral combinatory genetic and epigenetic explorations inside cells and among cells, populations and communities, expanding in different environments (migration), creating the stochastic variation required for evolutionary progress. Invasion refers to the process by which antibiotic resistance significantly increases in frequency in a given (invaded) environment, led by external invaders local multiplication and spread, or by endogenous conversion. Conversion occurs because of the spread of antibiotic resistance genes from an exogenous-resistant clone into an established (endogenous) bacterial clone(s) colonizing the environment; and/or because of dissemination of particular resistant genetic variants that emerged within an endogenous clonal population. Occupation of a given environment by a resistant variant means a permanent establishment of this organism in this environment, even in the absence of antibiotic selection. Specific interventions on emergence influence invasion, those acting on invasion also influence occupation and interventions on occupation determine emergence. Such interventions should be simultaneously applied, as they are not simple solutions to the complex problem of antibiotic resistant.

How has antibiotic resistance reached a planetary spread among all kind of environments? We cannot discard that other bacterial adaptive traits may also be spreading with high efficiency among bacterial populations, and that antibiotic resistance provides easy-to-detect phenotypes of obvious importance in public health. In any case, it seems reasonable to think that humans have–through the excessive use of antibiotics, biocides, and industrial pollution-accelerated the building-up and selection of genes and genetic platforms involved in antibiotic resistance. General factors derived from societal changes in human populations and the environment, including changes in land use (intensified human encroachment on natural environments, and globalization of planet biology—including human population growth; livestock and production methods; international travel or long distant trade of humans, animals, and vegetables, breakdown in public health infrastructure, and eventually geo-anthropological changes (such as global warming), microbial adaptation to drug or vaccine use or to new host species, might have also contributed to the global invasion by antibiotic-resistant bacteria. The rising consciousness about this multi-causal complexity requires a novel reconsideration of the priorities among possible interventions aiming to fight antibiotic resistance, including the possibility of influencing with complex treatments, restoration strategies, and may be with "drugs for the environment" the ecology and evolution of antibiotic-resistant bacteria [169, 170]. We should take seriously the possibilities of reducing antibiotic selection in the environment, as in fact in an environment polluted by low concentrations of antibiotics and potential human-pathogenic bacterial organisms serves as "training field" for the emergence and evolution of novel resistance traits.

5.3.6 Might Evolution of Antibiotic Resistance be Predicted?

The ultimate reason for any human scientific knowledge is the optimization or improvement of our current and future interactions with our environment. The reason for research in antibiotic resistance is, obviously, the possibility of disarming bacteria of their ability to counteract antibiotics. In a broader perspective, as was stated in the last paragraph, the aim is the preservation of a healthy microbial ecosystem surrounding humans. These objectives require mastering the evolutionary trajectories resulting in antibiotic resistance. Is that a feasible task? According to conventional scientific knowledge, evolution is essentially based on random processes which are exposed to an extremely large number of unexpected influences and is therefore essentially unpredictable. However, we generally act against this intuition with, for instance, hygienic procedures and implementation of antibiotic policies to prevent the development of antibiotic resistance are common practices in modern medicine. Indeed research in microbiological sciences applied to public health is currently based on the implicit belief that microbial variation and infectious diseases are predictable and therefore might (and should) be controlled before causing problems to mankind. If we are constantly seeking huge amounts of genomic and proteomic data from microbes, if we are building-up complex phylogenies, structural and mathematical models and developing advanced procedures based on systems biology to understand interactions between elements, it is only because we do not discard the possibility of preventing the emergence and dissemination of antibiotic-resistant microbial pathogens. Preventing this emergence and dissemination implies mastering the evolutionary trajectories of microbial pathogens, something that as previously stated goes against our conventional view of the process of evolution.

The main problem is the multi-causal origin of the spread of antibiotic resistance. To describe to a certain extent the main processes involved, a number of composite parameters should be analysed [171]. The main ones are certainly the following ones: (1) *contact rates*; this set of parameters refers to the probability that two particular elements involved in antibiotic resistance could be in close contact during a sufficient period of time, enabling potential interactions; for instance, susceptible and resistant cells, or plasmids carrying or not carrying particular genes; (2) *transfer rates*; this set of parameters refers to the probability that one of these elements moves into another element of the same or different hierarchical level; as a plasmid into a cell, or a gene into a plasmid; (3) *integration rates*; this set of parameters refers to the probability that one transferred unit could be stably maintained in coexistence with another element or assembled with it; (4) *replication* rates; this set of parameters refers to the probability that a particular element involved in antibiotic resistance will increase in copy number at a certain speed and reach certain final densities; (5) *diversification* rates; this set of parameters refers to the probability that a particular element (a clone, a plasmid, a gene) produces genetic variants at certain rates, and variants of these variants; and (6) *selection* rates; this final set of parameters refers to the probability that a particular element involved in antibiotic resistance might be replicating differentially than other units of the same hierarchical level as the result of the carriage of genes providing higher fitness.

The parametric space resulting from the above set of six rates measuring interactions of relevance in antibiotic resistance is certainly modified (even determined) by another group of parameters, the ecological parameters. These are environmental parameters whose changes might influence the above-mentioned rates. Among these parameters we can mention: density of colonized and colonizable hosts; population sizes of bacteria per host during colonization and infection; susceptibility to colonization of hosts, including age, nutrition, illness-facilitated colonization; frequency of between-host interactions (such as animal–human interaction); host natural and acquired immune response to colonizing organisms; ecological parameters of colonizable areas, including interaction with local microbiota and frequency and type of antibiotic-resistant commensals; migration and dispersal of colonized hosts; antibiotic exposure; overall density of antibiotic use, type of antibiotics and mode of action, dosage and duration of therapy, adherence to therapy, selective concentrations, antibiotic combinations; mode of transmission of resistant organisms; transmission rates between hosts (antibiotic treated and untreated, infected, and uninfected); time of contact between hosts; exposure to biocides; hygiene, infection control, sanitation; food, drinking-water and water body contamination, and host exposure; and environmental contamination by resistant organisms in soil, including sewage and water bodies.

Of course to obtain data to define this parametric field is an extremely complex task, certainly to be completed this century. We can of course with the powerful available bioinformatic technologies start to dissect this complexity. In the case of modular structures associated with resistance, the predictive process should be based on research about the "grammar of affinities" between modular elements. Techniques of comparative genomics have been used to infer functional associations between proteins based on common phylogenetic distributions, conserved gene neighbourhood, or gene fusions. The use of scoring-schemes in the building-up of networks describing possible associations between modules facilitates the prediction of novel functions [172, 173]. Similar types of methods could be developed to predict functional associations between modules involved in the emergence, expression, mobilization, or evolution of antibiotic resistance. A concern of these studies is their unaffordable complexity. Nevertheless, as in the case of mutation, genetic architectures based on modules might have an affordable complexity as they show reuse of alignments or circuit patterns which allow construction of complex adaptive systems by using common series of modules [174, 175]. From the perspective of a modular "genome system architecture" [176] it is possible to find in different organisms, plasmids, transposons, integrons or protein sequences such as recombinases, identical modules combined in different ways. The study of the corresponding linkage patterns has become critical for understand the evolution of evolvability [177]. Indeed MDR is the result of combinatorial genetic evolution [178, 179]. If it were possible to make comprehensive catalogues of modular functional units, combination of these modules in local alignments could be predicted that might fulfil the expected bacterial adaptation [180]. The building-up of comprehensive interconnected databases where modules could be stored in function of their combinations has been proposed [181]. These combinations probably depend on particular codes by which particular units are accepted (integrated) within others after introgressive events. For instance, (1) codes determining the compatibility of a acquired resistance gene with the functional structure of a cell or of mobile genetic elements; in fact the new character should be compatible with the metabolon, or selfish (a kind of individual) metabolism [182]; (2) codes determining the compatibility of resistance and virulence plasmids or ICEs with particular bacterial species and clones; (3) codes determining the compatibility of a particular bacterial resistant or virulent clone with specific microbiotic ensembles in particular hosts, including different animals. Unfortunately, we know very little about these codes, but such a knowledge is certainly needed for public health, to establish desirable surveillance and control measures assuring healthy relations between humans and animals, and the microbiosphere [183]. Bioinformatics (network genomics and proteomics) using

approaches like combinatorics, fuzzy logic models and principles learned from linguistics and semiotics may be able in the future to accomplish the task of finding a grammar of modular affinities [90, 176, 184] to approach one of the major objectives of all biological sciences: to be able to predict ("the topology of the possible" [185]) evolutionary trajectories of living beings. System biology [186, 187] and *ad-hoc* computational methods will take advantage of these data to establish predictions for antibiotic resistance [188].

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