12 The Evolution of Antibiotic Resistance

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12.1 Introduction

What is antibiotic resistance? This expression was obviously coined first in relation to medical microbiology and the therapy of infections. Antibiotic resistance refers to the property of bacteria which prevents the inhibition of their growth by antimicrobial agents used in the clinical setting. During the past decade many research, editorial, and review articles have focused on antibiotic resistance (Levy and Marshall, 2004; Pitout and Laupland, 2008; Livermore, 2009). The problem is dramatic in some countries (Vatopoulos, 2008) and especially worrying in highly pathogenic species such as *Mycobacterium tuberculosis* (Wright et al., 2009), methicillin-resistant *Staphylococcus aureus* (MRSA) (De Lencastre and Tomasz, 2008), *Acinetobacter baumanii* (Karageorgopoulos and Falagas, 2008), enterococci (Arias and Murray, 2008), or *Klebsiella pneumoniae* (Souli et al., 2008). Antibiotic resistance represents one of the best examples of natural selection, the basic process of evolutionary change, in action; and also one of the major hurdles in humankind's fight against infectious diseases. By taking a look at antibiotic resistance from a dual perspective, evolutionary and clinical, we hope to contribute to a better understanding of the principles and processes that result in the emergence of this undesirable character and to suggest strategies to, ideally, prevent or at least delay its extension. Human actions may not prevent evolution, but we can try to drive it through less damaging pathways.

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Antibiotic resistance can be "natural," when all the strains of the same bacterial species are resistant to a particular drug (also known as intrinsic resistance), or "acquired," when there are susceptible and resistant strains in the same species, with resistant strains having evolved from susceptible ones by selection after mutation or lateral genetic transfer events. It is of note that susceptible bacteria may have some type of "intrinsic resistance" along their life; for instance, high-density, slow-growing, stationary-phase bacteria are often refractory to inhibition by antimicrobial agents. This type of resistance is often qualified as "phenotypic" or "non-inheritable," as the organisms that are able to resist the drug under these circumstances give rise to a susceptible progeny under different conditions. Quite naturally, this view is controversial because when the original conditions are restored the resistant phenotype will occur again. The issue is even more complex, as "resistance" or "susceptibility" is not an absolute property.

A bacterial organism may be resistant to a particular concentration of an antibiotic and susceptible to a higher one. This is why we need breakpoints for defining susceptibility or resistance, based on minimal inhibitory concentration (MIC, the lowest amount of antibiotic able to inhibit bacterial visible growth) values. Clinical breakpoints consider a bacterial organism as resistant if its MIC is higher than the available concentration of the antibiotic at the site of infection, and/or higher than the concentration associated with favorable clinical outcomes. "Natural" (in contrast to clinical) breakpoints consider a bacterial organism as resistant if its MIC is significantly higher than the modal MIC of a collection of strains of the same bacterial species, thus considering resistance as an "abnormally higher" MIC, which indicates some kind of acquired genetic change.

Antibiotic resistance and MIC values have been consistently rising for many bacterial species and populations, even nonpathogenic ones, since the start of the industrial production of antimicrobial agents. The total annual production of antibiotics can be estimated between 100,000 and 200,000 tons and about half of them are not used in humans (mostly in farming) (Gootz, 2010). Considering that most antibiotics act at concentrations close to 1 μg/mL, such amount of antimicrobials is enough to cover the entire surface of the Earth with inhibitory concentrations; in other words, they are capable of altering the population genetic structure of microbes. Moreover, antibiotics are not easily removed from the environment, and some of them can remain active for extended periods of time. The release of antibiotics is probably one of the major anthropogenic effects on the microbiosphere, altering microbial systems. Part of these alterations are predictable, such as extended antibiotic resistance, but unpredictable effects are most likely to occur, as changes in the interactions between microbes or with animals, plants, or influencing basic cycles of life in the biosphere (Baquero, 2009a,b; Martínez, 2009).

The use of industrial antibiotics in human and veterinary medicine, agriculture, farming, and other areas converges to a single, cooperative effect, changing bacterial ecology not only in different environments but also in the *common* environment. This effect can be observed as selection for antibiotic-resistant organisms and a faster evolution of antibiotic resistance. The main problem is the existing connectivity between all environments, human, farming, and agricultural, so that

the antibiotic-derived effects in one of them has consequences in all the others. The connection between different environments with regard to the undesirable consequences of antibiotic resistance occurs essentially in two ways, which will be considered in the next paragraphs.

First, dispersal and migration of biological units, such as bacterial communities, bacterial clones, mobile genetic elements (MGEs), and, in general, genes, play a major role in antibiotic resistance. Second, there is also dispersal of antimicrobial ecotoxic agents, which results in the production of selective mixed gradients and stressor effects, and in an acceleration of microbial evolutionary rates. The combination of migration of antibiotics and antibiotic-resistance biological units results in evolutionary activating interactions that occur in four main genetic reactors: (i) the intestinal microbiota of humans and animals; (ii) the highly antibiotic-exposed areas with high rates of bacterial transmission, such as hospitals (particularly newborn wards and intensive-care units), (iii) wastewater, effluents, and sewage treatment plants, and (iv) soil, sediments, surface and ground waters (Baquero et al., 2008), all of which contribute to the escalation of the emergence and spread of antimicrobial resistance.

The most evident (visible) threat of antimicrobial resistance for humankind is the failure of therapy against infectious diseases. The decrease in the incidence of infectious diseases in the Western world started in the beginning of the nineteenth century, by reasons related to social progress, better nutrition, and housing and hygienic procedures, but in the absence of antibiotics. Nevertheless, the discovery and subsequent industrial production of antimicrobials between 1935 and 1960 was followed by a significant reduction in the morbidity and mortality by infections, particularly the more severe ones, and has probably contributed to the increase in the expected duration of lifetime of human populations. At the same time, antibiotics facilitated the progress in Medicine at large, allowing interventions (complex surgery, intensive-care units, immunosuppressive and anti-cancer chemotherapy, or transplantation) that expose the impaired host to both pathogenic and opportunistic bacterial infections.

If antibiotic resistance were surpassing a threshold limit, the consequences on the current standards of hospital-based medicine (including long-term-care facilities for the elderly) could become severely compromised. With the emergence of multi-resistant Gram-positive organisms, such as MRSA or *Enterococcus faecium*, or Gram-negatives, such as pan-resistant *Escherichia coli*, *Klebsiella pneumoniae*, or *Pseudomonas aeruginosa* producing extended-spectrum β-lactamases (ESBL) and carbapenemases are currently very close to such a threshold. A transient equilibrium was reached from 1950 to 1980 because resistance was counteracted by the continued discovery of novel antimicrobial agents active on resistant strains. Unfortunately, during the last quarter of the twentieth century no significant advances occurred in this field as a result of the interest of a number of pharmaceutical companies in investing more in chronic, non-curable diseases. In our days, resistance to the newest antibiotics continues evolving mostly on the bases of the old genetic mobile structures (plasmids, transposons, integrons) that became prevalent by the selective effect of the old antibiotics. The effect that the anthropogenic

release of antibiotics has already caused on the genetic structure of bacterial populations is probably irreversible and will influence the evolutionary future of microbes on Earth.

Cleaning nature of this resistance gene pool is impossible. The best we can do is trying to control the emergence, selection, and spread of antibiotic-resistance genes in bacterial organisms interacting with humans, animals, or plants. The classical strategies for controlling the emergence of resistance are based on the reduction of chronic antibiotic-promoted bacterial mutagenic-stress associated with low dosages, the use of combinations of drugs, early-intensive therapy, maintaining low the bacterial density, and, more recently, the surveillance of hypermutable organisms and the suppression of phenotypic resistance. A number of these strategies have been explored by population and mathematical modeling (Levin et al., 2000; Levin, 2001; Bergstrom et al., 2004). Controlling the selection of antibiotic resistance is a major practical goal, which can be addressed again by the development of novel anti-infective drugs and the appropriate use of antibiotics, avoiding low dosages able to select low-level mutations serving as stepping-stones for high-level resistance. A classic approach to prevent the spread of antibiotic resistance is based on general hygiene and containment (infection control) measures, for instance by decreasing contacts between patients contaminated (infected or carriers) with resistant bacteria and non-contaminated ones.

Unfortunately, these measures are becoming increasingly insufficient in the current global landscape of antibiotic resistance. Avoiding the emergence of resistance in the individual patient is obviously important for the individual, but has minimal effects at the community level. The efficacy of classical ways of controlling selection and spread is inversely proportional to the density and penetration of resistant organisms and their MGEs in particular environments. Measures that might be successful in the early stages of resistance development, or in hospitals or countries with low rates of antibiotic resistance, are worthless in areas where resistance is already well established. Even in low antibiotic-resistance polluted areas, such as Sweden, recent studies have shown that a 2-year discontinuation in the use of trimethoprim did not reduce significantly the *E. coli* resistance rates to this compound (Sundqvist et al., 2010). This was probably due to the dispersion of trimethoprim-resistance genes in a multiplicity of bacterial organisms and MGEs frequently harboring other resistance determinants, thus assuring co-selection of *dfr* genes with other resistance genes.

Some regions of the world are densely polluted with antibiotic resistance. In a global world, sooner or later, resistance originated in these "source of resistance" areas will invade still clean environments. Resistant organisms are constantly diluted and potentially extinguish in competition with constant immigration of susceptible bacteria in local environments, but such a trend might collapse by the increase of resistant populations. Moreover, the success of resistant organisms will contribute to the constant accumulation in the bacterial world of genetic platforms and vehicles able to efficiently recruit and spread novel resistance genes. Antibiotic resistance increases bacterial evolvability; resistance calls for more resistance, in a phenomenon described as "genetic capitalism" (Baquero, 2004). In other words,

resistance might be reversible when rare; if frequent, reversibility is not to be expected.

12.2 Mechanisms and Sources of Antibiotic Resistance

To produce an effect in a bacterial cell, an antibiotic has to cross different cell envelopes, in some occasions has to be activated by bacterial enzymes, and reach its target at a concentration high enough to allow a successful interaction and the inhibition of bacterial growth or killing (Figure 12.1A). Resistance can thus be achieved either if the antibiotic concentration reaching the target is too low or if the interaction between the antibiotic and the target is not efficient enough to produce the inhibition of bacterial growth. This includes intrinsic and acquired resistance (Figure 12.1).

The most classical mechanisms of intrinsic resistance are the absence of the target and a reduced permeability to a given antibiotic. These two mechanisms are passive systems of resistance. However, bacterial populations also present active mechanisms of resistance based on the detoxification of the antibiotic. These include chromosomally encoded antibiotic-inactivating enzymes and multidrug (MDR) efflux pumps. More recently, the analysis of comprehensive libraries of mutants from *E. coli* (Tamae et al., 2008) and *P. aeruginosa* (Breidenstein et al.,

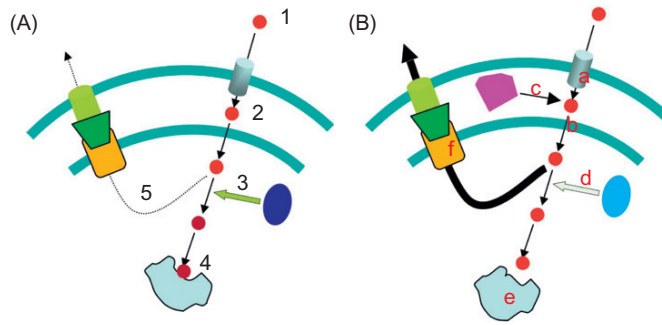

Figure 12.1 Basic mechanisms of antibiotic action and resistance. In order to inhibit bacterial growth an antibiotic requires to successfully interact with its target at concentrations high enough for inhibiting its activity. For this (A), the antibiotic (1) requires to traverse cellular envelopes (2), in some occasions to be activated by an intracellular enzyme (3), and reach its target (4). The activity of constitutively expressed MDR efflux pumps (5) can decrease the intracellular concentration of the antibiotic. As shown in (B), resistance is achieved by interfering with this pathway, either by changes that impede the entrance of the antibiotic (a, b), by the activity of antibiotic-inactivating enzymes (c) or overexpression of MDR efflux pumps (f) that reduce the effective intracellular concentration, or by mutations in the enzyme that activates the pre-antibiotic (d) or in its target (e), which preclude target/antibiotic interactions.

2008; Fajardo et al., 2008; Dotsch et al., 2009) has demonstrated that several genes participate in the intrinsic resistance phenotype of a bacterial species. This suggests that intrinsic resistance is not just a consequence of the adaptation of a bacterial species to the presence of a given antibiotic, but rather a phenotypic consequence of the general physiological characteristics of each species.

Contrary to intrinsic resistance, which is an ancient phenotype of bacterial populations, acquired resistance is the consequence of adaptive evolution to the recent selective pressure exerted by the utilization of antibiotics by humankind (Martinez, 2009). Resistance can be achieved by avoiding the activity of the antibiotic at different levels (Figure 12.1B). These include changes in bacterial targets, which prevent the efficient action of the antibiotic, and the reduction of the effective intracellular concentration of the antibiotic, achieved by changes in the permeability of the cell envelopes (including a reduced expression of specific membrane transporters), by the expression of antibiotic-inactivating enzymes or MDR efflux pumps capable of efficiently expelling the antibiotics out or, in the case of intracellularly activated pre-antibiotics, because the enzyme involved in their activation is not expressed or is not active as a consequence of mutations.

A formerly susceptible organism can acquire resistance by two main genetic mechanisms: mutation (Martinez and Baquero, 2000) or acquisition of foreign DNA (Davies, 1994). Mutation is the major cause in originating resistant bacterial pathogens during infections in the absence of a donor of antibiotic-resistance genes (Macia et al., 2005). Mutations involved in the development of resistance produce structural changes in the targets (for instance, quinolone resistance due to mutations in genes coding for bacterial topoisomerases; Piddock, 1999), in the enzymes that activate the pre-antibiotic (for instance, in the resistance to isoniazid due to mutations in the *Mycobacterium* catalase gene; Zhang et al., 1992), or in antibiotic transporters. Mutations in regulatory elements are also relevant for acquiring resistance. They act by changing the level of expression of antibiotic transporters (for instance, the porin OprD2 that transports imipenem inside *P. aeruginosa* and is not expressed in imipenem-resistant mutants; Yoneyama and Nakae, 1993) or by increasing the expression of antibiotic-detoxifying systems such as chromosomally encoded antibiotic-inactivating enzymes (Nelson and Elisha, 1999) and MDR efflux pumps (Martinez et al., 2009b). As we will see later, mutation is also important for the evolution of antibiotic-resistance genes acquired by bacterial pathogens through horizontal gene transfer (HGT).

Resistance can be also achieved as a consequence of the incorporation of DNA from other bacteria. In some occasions, this DNA recombines with homologous genes present in the chromosome of the new host, rendering novel mosaic genes that make the host resistant to antibiotics (for instance, the formation of recombinant penicillin-binding proteins; Spratt et al., 1992; Sibold et al., 1994). In other occasions, resistance is attained by the acquisition of an element that confers resistance on its own (antibiotic-resistance genes) (Davies, 1997). Given that bacterial pathogens were susceptible to antibiotics prior to their use as therapeutic agents for treating infections (Datta and Hughes, 1983), one intriguing question concerns the origin of antibiotic-resistance determinants.

Since natural (i.e., non-synthetic) antibiotics are produced by environmental microorganisms (Waksman and Woodruff, 1940), it was proposed early that antibiotic-producing microorganisms would be the most likely source of resistance determinants (Benveniste and Davies, 1973). The rationality of this proposal comes from the need of producers to protect themselves from the activity of their own antimicrobials. It was proposed that the actual source of resistance could be the DNA from antibiotic producers that might contaminate antibiotic preparations, which could be incorporated by bacterial pathogens simultaneously to treatment (Webb and Davies, 1993). This origin from antibiotic producers might be true on occasion, and indeed a recent search of resistance elements in *Actinomycetes*, the most important group of antibiotic producers, demonstrates that they harbor in their genomes a very large number of genes whose expression can trigger resistance in other hosts (D'Acosta et al., 2006). Nevertheless, in spite of the efforts at tracking the origin of resistance genes currently present in human pathogens, yet little is known about the source of these determinants, and in most occasions the comparison of resistance genes present in human pathogens to those located in the chromosomes of the microorganisms selected by the pharmaceutical industry for producing antibiotics show that they are not exactly the same but rather belong to the same structural family, thus indicating a phylogenetic relationship but not a direct origin.

Notably, a direct origin of some specific resistance genes has been demonstrated for bacterial species that do not produce antibiotics. This is the case for Qnr determinants, which contribute to plasmid-acquired low-level resistance to quinolones in Enterobacteriaceae (Robicsek et al., 2006b). It is worth mentioning that quinolones are synthetic antibiotics, so that it was proposed that resistance could be achieved only by mutations and that the existence of quinolone-resistance genes that could be acquired by human pathogens would be unlikely. Contrary to this hypothesis, the existence of plasmids carrying quinolone-resistance genes was described in 1998 (Martinez-Martinez et al., 1998) and, as stated above, these plasmids are currently disseminated among Enterobacteriaceae. The search for *qnr* elements in the chromosomes of fully sequenced bacteria has shown that these determinants are mainly present in aquatic bacteria, which do not produce antibiotics (Sanchez et al., 2008). As these genes are conserved among members of the same species and the level of synteny of the flanking regions in the chromosome is high, this indicates that the analyzed aquatic bacteria have not acquired those elements from other bacteria but rather those microorganisms are the source of Qnr determinants. Indeed, the origin of the *qnrA1* gene, the most abundant in Enterobacteriaceae plasmids, is *Shewanella algae* (Poirel et al., 2005). Similarly, *Kluyvera ascorbata*, a non-producer organism, is the most likely origin of the β-lactamases belonging to the CTX-M family (Humeniuk et al., 2002).

The functional role of resistance genes in producers seems clear: they might be detoxification elements needed to avoid the activity of the antibiotic. However, their function in non-producers is less apparent. It can be postulated that these elements might serve to resist the activity of inhibitory compounds produced by competitors in complex microbial ecosystems. As we will describe later, this might be

the function of some of these resistance elements. However, on other occasions, these elements have not evolved to specifically counteract the activity of antibiotic producers. For instance, Enterobacteriaceae harbor chromosomal β-lactamases (Lindberg and Normark, 1986), which have been present in their genomes for several hundred million years. However, the natural habitat of these bacterial species is the gut, an ecosystem that does not contain β-lactam producers and, thus, did not contain β-lactam antibiotics until the recent use of these compounds for the treatment of infections. Similarly, quinolones are among the most frequent substrates of MDR efflux pumps (Alonso et al., 1999) despite the synthetic origin of these antibiotics, which implies that bacterial populations have not been exposed to their action until recently (in evolutionary terms). A suitable hypothesis for explaining the origin of these elements might be that they have been selected during bacterial evolution to play other roles than resistance (Fajardo et al., 2008), but their natural substrates present structural similarities to antibiotics currently used for therapy, in such a way that they can detoxify bacteria from these drugs even though this is not their original physiological function.

For instance, all bacterial species harbor in their genomes genes coding for MDR efflux pumps. These genes are highly conserved (the same MDR elements are present in all the strains of a given bacterial species) and redundant (bacterial species usually harbor several different MDR efflux pumps), indicating that they are ancient elements relevant for bacterial physiology (Martinez et al., 2009b). Given their genomic redundancy and their overlap in substrate usage, it is unlikely that the original function of these elements would be resistance to the drugs currently used in the clinical setting. This is the case of AcrAB-TolC, the major MDR efflux pump in *E. coli* and *Salmonella*. This element can extrude, besides antibiotics, bile salts (Thanassi et al., 1997), which are toxic compounds present in the natural ecosystem of these bacterial species, the gut. Similarly, it has been described that other MDR efflux pumps (that contribute to antibiotic resistance) can have a primary role in the trafficking of bacterial signals (Kohler et al., 2001), in the bacterial response to plant-produced signals (Burse et al., 2004; Maggiorani Valecillos et al., 2006), in the detoxification of intracellular toxic intermediary metabolites (Aendekerk et al., 2002, 2005) or in the response to non-antibiotic bacterial inhibitors such as heavy metals (Nies, 2003) or solvents (Ramos et al., 2002).

All this indicates that the universe of elements that can confer resistance to a heterologous host upon their transfer by HGT is larger than previously thought. As an example, the study of the **resistome** of the human gut microbiota (Sommer et al., 2009) indicates the existence of a large number of elements that are not disseminated among human pathogens and that can confer resistance despite the fact that this ecosystem does not contain the regular producers of antibiotics, Actinomyces. The potential universe of resistance genes will thus include those that first evolved to confer resistance (for instance, in producers) but also those that can recognize the antibiotic even though their original substrate (and thus their functional role) was a different one.

Given the large number of resistance genes present in natural ecosystems, it is paradoxical that, in comparison, the number and variability of HGT-acquired

resistance determinants currently present in human bacterial pathogens are relatively low. This might indicate that there are some restrictions for the transfer of a potential resistance element to a human pathogen. The first impediment would be ecological connectivity. The presence of resistance elements has been demonstrated in bacteria from the deep terrestrial subsurface (Brown and Balkwill, 2009) and in the deep Greenland ice core (Miteva et al., 2004), where the presence of human pathogens is not expected. The probability of transfer of these elements to human pathogens will thus be very low, but chances will increase for bacteria whose natural habitats are closer to those of human pathogens. One example of this type or "reactors" for resistance are wastewater treatment plants, where human-linked microbiota (recipients of resistance genes) can get in contact with environmental microorganisms (potential donors) in the presence of residues of antibiotics which act as selecting agents (Baquero et al., 2008).

A second obstacle for the transfer of a resistance gene will be its integration into an efficient dissemination vector or into a bacterial epidemic clone, which will allow a fast spread for the resistance determinant (Baquero, 2004; Martinez et al., 2007).

The third obstacle consists of the fitness costs associated with the acquisition of resistance (Andersson, 2006). It is generally accepted that the development of resistance by formerly susceptible bacteria might confer a metabolic burden such that resistant populations might be outcompeted by susceptible ones in the absence of antibiotic selective pressure. It is worth mentioning that, unless the fitness costs are unaffordable, their relevance during antibiotic treatment will be negligible because in these conditions being resistant is a prerequisite for producing an efficient infection (Martinez and Baquero, 2002). Nevertheless, fitness costs may be highly relevant for the persistence and spread of resistance in the absence of selection (for instance, in nonclinical, natural ecosystems). It has been described that the fitness costs associated to the development of resistance to a specific antibiotic might be different depending on the involved mechanisms (Balsalobre and de la Campa, 2008). Furthermore, fitness costs can be compensated by secondary mutations in the bacterial genome (Bjorkman et al., 2000; Paulander et al., 2007; Lofmark et al., 2008). The acquisition of a resistance-determinant by a gene-recruitment element such as an integron, which might harbor other resistance elements (for instance, heavy metals), might allow the maintenance of resistance by co-selection mechanisms. Finally, the incorporation of a resistance-determinant into a plasmid encoding toxin-antitoxin systems allows the persistence of resistance even in the absence of selective pressure (Moritz and Hergenrother, 2007).

Overcoming all these biological and ecological obstacles is almost a necessary condition for the establishment of a resistance-determinant in a bacterial population. Nevertheless, several resistance-determinants besides those already disseminated among human pathogens might fulfill the requirements to overcome those obstacles. However, they are not currently disseminated. To explain this restriction further, **founder effects** must be considered (Martinez et al., 2009a). If one resistance element enters a formerly susceptible population through an efficient vector (or clone) and does not confer a high-fitness cost, it will soon spread in

environments, like clinical settings, with a high antibiotic load. Once this element is established, the antibiotic no longer exerts a selective pressure because the bacteria are already resistant to it, so that the acquisition of a new resistance-determinant will not represent an adaptive advantage. As a result, human antibiotic usage has rendered a strong increase in the prevalence of a few resistance elements that were previously present in the chromosomes of environmental microorganisms and are now located in gene-transfer units spreading not just in bacteria in clinical settings but also in environmental ecosystems.

The release of human pathogens harboring gene-transfer units containing resistance elements, eventually simultaneously with antibiotic-containing wastes, might have a deep impact on the evolution of the microbiota from natural ecosystems, and this can influence also the evolution of clinically relevant mechanisms of antibiotic resistance (Baquero et al., 2008). As an example, the same antibiotic-resistance elements currently present in human pathogens can be found in wild animals (Livermore et al., 2001; Pallecchi et al., 2008) or in environmental locations without a history of antibiotic pollution (Pei et al., 2006). Furthermore, the study of historical soils has demonstrated that the introduction of antibiotics has produced an increase in the prevalence of specific resistance determinants in environmental, nonclinical ecosystems (Knapp et al., 2010).

The recent use (in evolutionary terms) of antibiotics by humankind has produced a strong enrichment in the distribution of a few specific antibiotic-resistance elements in clinical and nonclinical ecosystems. The impact of this enrichment in specific genes, and eventually bacterial clones, on the composition and activity of the microbiosphere remains to be fully understood. Given that natural ecosystems are the source of resistance genes (Martinez, 2008) and the reservoirs for their maintenance (Simoes et al., 2010), more studies on the ecological behavior of resistance in nonclinical habitats are required to unveil how these changes might impact on the acquisition of antibiotic resistance by human pathogens.

12.3 Evolution of Antibiotic-Resistance Genes

12.3.1 Antibiotic-Resistance Genes as Targets of Evolution

Antibiotic resistance is not only a clinical problem. It also represents a unique opportunity for observing evolution almost in real time, and therefore a meeting point between clinical and evolutionary microbiologists. The evolution of antibiotic resistance is a consequence of the selection of resistant organisms. Thanks to the acquisition of antibiotic-resistance genes, these bacteria are able to express a set of functions (resistance phenotypes) in a particular physiological and ecological context. On the other hand, the evolution of antibiotic resistance does not depend only on genes encoding mechanisms of resistance. The acquisition, expression, variability, and persistence of these genes depend on the genetic environment in which they are hosted, as integrons, transposons, plasmids, phages, or bacterial clones.

The evolution of resistance genes is therefore dependent on cell physiology and ecology. In consequence, antibiotic selection of antibiotic-resistant organisms implies the selection of organisms with particular physiological or ecological abilities.

A classic theoretical problem in evolutionary biology is whether or not genes are the units of selection. According to the preceding paragraph, and the conventional wisdom in evolution, selection acts on organisms that exhibit particular (selectable) phenotypes, and genes encoding those phenotypes will be selected by second-order processes of selection on the organisms that harbor them. A different (more reductionist) view was proposed by Dawkins (1976), who defended the preeminence of the gene as a selfish evolutionary element, an idea derived from the differentiation between replicators and vehicles. This view is weakened in the microbial world, as the "vehicles" (the organisms) are not really as perishable as higher sexual organisms are. Indeed, only HGT restores the importance of gene evolution in the microbial world. Genes may spread among bacterial populations and therefore are able to "replicate" independently from their vehicles. Indeed, genes involved in antibiotic resistance frequently migrate and spread among commensal and pathogenic organisms, and therefore have an evolutionary history which is independent of that of their bacterial hosts. In the following paragraphs we will illustrate a number of features related to the evolution of antibiotic-resistance genes, focusing only on those genes able to provide resistance phenotypes, leaving carrier genetic structures (such as integrons, transposons, phages, or plasmids) out of the scope of this review.

After the initial views that antibiotic-resistance genes had their origin in the environment (Benveniste and Davies, 1973) and that producers spread antibiotic-resistance genes to bacterial pathogens of humans (Marshall et al., 1998), it was accepted that genes encoding mechanisms of resistance arise in potentially any bacteria, because the ancestral precursors are present in all of them, in most cases as house-keeping genes involved in the physiological functions required for daily bacterial life. There are well-documented examples, such as β-lactamases, proteins capable of inactivating penicillin and cephalosporins, derived from protein-binding proteins (PBPs), which are essential in the construction of the peptidoglycan layer (Massova and Mobashery, 1998) or the essential Ser/Thr/Tyr protein kinases, the ancestral proteins of aminoglycoside or macrolide inactivating enzymes (Shakya and Wrigh, 2010). More recently, the sequencing of many complete bacterial genomes has provided a more complex scenario, showing that bacteria can have multiple alternative pathways to develop low levels of antibiotic resistance (Fajardo et al., 2008), thus leading to the concept of resistome (Wright, 2007). Examples such as GadA and GadB proteins (glutamate decarboxylase) as well as AmpC and HdeB, proteins that increase ampicillin resistance in *E. coli* (Adam et al., 2008), show the possibilities of different evolutionary pathways for developing antibiotic resistance in bacteria.

Therefore, resistant phenotypes can occur and even evolve in the absence of antibiotic selection; conversely, antibiotics may influence the evolution of bacterial functions associated to the adaptation to particular environments. In any case, it is essential to understand that there is a wealth of potential mechanisms of resistance

contained in bacterial chromosomes and in MGEs. In this section we will illustrate a number of issues related to the evolution of genes directly involved in antibiotic-resistance phenotypes.

The main mechanisms of gene variation leading to variation and diversification of antibiotic-resistance genes are mutation, recombination, and amplification. The frequency of these mechanisms is variable in normal populations, being typically from 10^{-9} to 10^{-6} in the case of mutation, from 10^{-7} to 10^{-13} for recombination, and from 10^{-5} to 10^{-2} for tandem gene amplification. Note that considering the large sizes of bacterial populations (normally exceeding 10^{8} cells/mL in their niches) these mechanisms of variation should be sufficient to provide enough variants of potential adaptive value in the presence of antibiotics. This is not necessarily the case if these populations are reduced during the infective process by innate or acquired immunity, by antimicrobial drugs, or during tissue-to-tissue or host-to-host transmission bottlenecks. In these cases population sizes might be insufficient to provide resistant mutants.

Bacteria with increased mutation rates are usually known as mutators. They present 10- to 1000-fold more chances of introducing changes in their DNA sequence during each replication cycle and therefore have a high probability of generating selectively advantageous mutations giving rise to the acquisition of antimicrobial resistance (Blázquez, 2003). Hypermutation is generally a consequence of a defective mismatch-repair system; as expected, mutators are easily selected when bacterial populations are exposed to recurrent selection pressures (such as during antibiotic treatments) (Mao et al., 1997). Mutators have been described, for example, in clinical strains of *S. aureus*, *S. pneumoniae*, *H. influenzae*, *E. coli*, *P. aeruginosa*, or *S. maltophilia* (Oliver et al., 2000; Morosini et al., 2003; Baquero et al., 2004; Turrientes et al., 2010). Mutators have been used in order to experimentally predict the emergence and selection of resistant variants (Galán et al., 2003; Novais et al., 2008; Stepanova et al., 2008), even though other methods are even more powerful to generate diversity (Orencia et al., 2001; Rasila et al., 2009). If predictions based on the use of **mutator strains** can provide insights about the type of variants conferring resistance to a particular antibiotic, they do not give us certainty about which of these variants will be more successful in the real world. This suggests that other factors apart from single antibiotic selective pressure also affect the evolution of the resistance phenotype. For instance, it has been recently demonstrated that the huge diversity of CTX-M β-lactamases can only be explained by the simultaneous exposure to two antibiotics, cefotaxime and ceftazidime, **genetic drift** also playing a relevant role in some stages of the process (Novais et al., 2010).

Antibiotics might act as accelerators of mutation-based evolution of resistance (Couce and Bláquez, 2009) not only by selecting mutator strains (Oliver et al., 2000), but also inducing intracellular redox imbalance, thus increasing intracellular superoxides. Accumulation of hydroxyl radicals (ROS formation) is highly toxic for the cell, and has a potent mutagenic power following the induction of the SOS response resulting from DNA damage. Elicited SOS-repair processes also increase the expression of error-prone polymerases such as polymerases IV (DinB) and V

(UmuCD), which present 100-fold less fidelity than the canonical polymerase II (PolB). In consequence, bactericidal antibiotics (bacteriostatic antibiotics are not inducers of hydroxyl radicals) have a double effect, inducing a process leading to cellular death by blocking their target (PBPs, DNA gyrase, etc.) while promoting the generation of genetic diversity and, therefore, of antibiotic resistance. An elegant model is the fluoroquinolone-resistance mediated by *qnr* genes: treatment with ciprofloxacin induces the SOS response, increasing the expression of error-prone polymerases (in a similar way to β-lactams or aminoglycosides), and promotes the cleavage of the LexA protein, a negative repressor of the *qnrB2* gene, thus leading to QnrB overexpression. QnrB binds to DNA gyrase, protecting it from quinolone inhibition (Da Re et al., 2009).

Recombination is a powerful mechanism for the evolution of antibiotic-resistance genes, particularly relevant for organisms able to exchange DNA by transformation, such as *S. pneumoniae*, *Neisseria meningitidis*, or *H. influenzae*, the typical example being recombination between sequences of *pbp* genes (penicillin-binding proteins) leading to different β-lactam resistance phenotypes in *S. pneumoniae* (Brückner et al. 2004). In this same species, hyper-recombinant strains have been associated to the generation of antibiotic resistance (Hanage et al., 2009). We should admit that the extent by which transformation and subsequent gene recombination occurs in many bacterial organisms is widely unknown; for instance, population biology studies on *Enterococcus faecalis* indicate high frequency of recombination among clones, despite the fact that transformation has not been documented in this species (Ruiz-Garbajosa et al., 2006). Indeed intraorganismal gene recombination is also a powerful mechanism for antibiotic resistance gene evolution, particularly relevant to rapidly spread adaptive mutations within a genome when they occur in a copy of otherwise repeated homologous genes. This phenomenon, known as gene conversion (Prammananan et al., 1999), increases for instance the efficiency of antibiotic-resistance mutations within *rrn* genes.

Finally, tandem gene amplification has been suggested as a possibility for evolving novel antibiotic-resistance genes. Amplification might immediately provide an adaptive advantage (increased gene dosage might increase the level of resistance protein), and later, eventually, one of the copies might serve as basis for further gene evolution without the risk of losing the ancient function (Pettersson et al., 2009; Kugelberg et al., 2010). Interestingly, all these mechanisms of variation (mutation, recombination, amplification) can increase under antibiotic stress at sub-inhibitory concentrations (López and Blázquez, 2009).

Resistance genes are frequently able to further evolve under variable antibiotic selection, allowing bacteria to grow when exposed to increased antibiotic concentrations, or enlarging the number of antibiotics within a family to which they provide protection. A good example is illustrated by a mutation in position 83 (Ser-83) of the A subunit of the DNA gyrase which provides a ciprofloxacin MIC of about 1 μg/mL in clinical isolates; a double change in Ser-83 and Asp-87 was found to increase MICs reaching values equal or higher than 8 μg/mL (Vila et al., 1994). Another example is provided by the cumulative mutational events driving the diversification of CTX-M β-lactamases (Coque et al., 2008; Novais et al., 2010).

12.3.2 Potential Evolution of the Enzymes Involved in Antibiotic Resistance

From a structural point of view, proteins involved in antibiotic resistance frequently show a high molecular plasticity, easily generating variants with an enlarged spectrum of action for new drugs within a chemical family, particularly under intense exposure. One of the first evidences on the functional versatility of antibiotic-resistance genes was described using TEM-1 β-lactamase. It was found that only 43 out of the 263 amino acid residues did not tolerate substitutions without loss of function (Huang et al., 1996). Large numbers of different variants were selected when bacteria carrying TEM-1 were exposed to cefotaxime (a synthetic β-lactam antibiotic active against TEM-1 producing organisms) (Zaccolo and Gherardi, 1999). This plasticity is also common in other determinants conferring resistance to other families of antibiotics, such as tetracyclines (Thaker et al., 2010) or aminoglycosides (Smith and Baker, 2002). However, β-lactamases are still the best model to understand the evolutionary potential of antibiotic-resistance elements (Bush and Jacoby, 2010), as β-lactams are the most extensively used antibiotics in the clinical setting (Goossens, 2009) and the family for which largest number of chemical molecules have been developed (due to their low level of toxicity and relatively low costs of production). Simultaneous strong selective pressure and changing selector (different β-lactam) has thus allowed the evolutionary radiation of β-lactamases during decades.

From 1940, when penicillin was commercialized, to 2007, when the last carbapenem (doripenem) was approved, β-lactamases have been in continuous evolution. In 1944 the *Staphylococcus aureus* PC1 penicillinase was the first enzyme known to confer resistance to the natural compound, penicillin. Subsequently, plasmid-mediated broad-spectrum β-lactamases (TEM-1 β-lactamase) were found in 1963 and ESBL in 1983 (Sirot et al., 1987), 5 years after cefotaxime was commercialized. The inhibitor-resistant TEM-type or SHV-type variant β-lactamases (IRT or IRS) were detected in 1992 (Blázquez et al., 1993), 8 years after the introduction of amoxicillin-clavulanate in the clinical setting. Plasmid-mediated carbapenemases (both metallo-β-lactamases and serine-β-lactamases) were detected in 1999 and 2003 (Lauretti et al., 1999; Smith Moland et al., 2003), when the consumption of carbapenems increased. Now almost 900 different β-lactamases are known. They are divided in four groups (A−D) according to their enzymatic properties and evolutionary relationships; class A β-lactamases being the most widely distributed. In fact, the number of variant class A β-lactamases with clinical importance is enormous and they include about 190 TEM and OXA-variants, 135 SHV variants, 100 CTX-M variants, or 35 carbapenemases. It is important to remark that, although the genes at the evolutionary root of these groups of β-lactamases had an origin in environmental bacteria, their subsequent mutation-driven evolution has likely taken place in clinical environments as the consequence of strong selection pressure by changing β-lactams. Among TEM variants, 66 amino acid positions (∼25% of all) have changed at least once. Nevertheless, this overwhelming diversity in antibiotic-resistance protein evolution follows only a few mutational pathways (Weinreich

et al., 2006), as either some mutations are antagonistic when they are present in the same enzyme, or some mutations provide a phenotypic advantage only in specific backgrounds (sign epistasis) (Poelwijk et al., 2007).

An example of the first case is the simultaneous presence of P167S and D240G changes, which are involved in extended-spectrum activity of the CTX-M β-lactamases toward hydrolyzing ceftazidime efficiently, and both confer a lower hydrolytic activity against ceftazidime than each change alone (Novais et al., 2008). When the mutational pathway toward the highest cefotaxime hydrolytic activity was reconstructed by site-directed mutagenesis, some mutations yielded either an increase or decrease in resistance depending on the previous mutations, indicating that there is an order in the selection of mutations (Novais et al., 2010). Also, in the case of TEM β-lactamase, the M182T change conferred positive effects on the hydrolytic activity of TEM β-lactamase on eight occasions, with five neutral and three negative (Weinreich et al., 2006).

The plasticity of the antibiotic-resistance gene plays an important role in the selection of antibiotic resistance, but such plasticity may have a limit related to the possible loss of function. In any case, in a landscape dominated by HGT, clonal dispersal into different hosts, and under a highly selective exposure, a rapid diversification of antibiotic-resistance genes is expected, as it has recently occurred with plasmid-carried fosfomycin-resistance or quinolone-resistance genes (Martinez-Martinez et al., 1998; Wachino et al., 2010). When the local prevalence of determinants of resistance is high, two or more determinants are likely to be simultaneously present in the same strain, thus favoring recombination events originating hybrid proteins (Barlow et al., 2009). These hybrid enzymes would be selected if they confer selective advantages, such as increasing MIC values to antibiotics, reducing the fitness cost of resistance, or increasing the spectrum of activity.

Mosaic genes have been described among tetracycline-resistant determinants, *tet* genes, especially between *tet*(O) and *tet*(W) in Firmicutes (Patterson et al., 2007) showing higher protection against tetracycline than non-recombinant *tet* genes (Stanton and Humphrey, 2003). Recently, a hybrid β-lactamase (609 amino acids) with two active centers, resulting from the fusion between class C (346 amino acids) and class D (253 amino acids) β-lactamases (Allen et al., 2009), has been described. Probably the best examples known are the hybrid bifunctional enzymes of aminoglycoside resistance AAC(6′)/APH(2′), an enzyme able to perform acetylation and phosphorylation, and ANT(3″)-Ii/AAC(6′)-IId, an enzyme that catalyzes acetylation and adenylation reactions (Zhang et al., 2009). However, the most worrying discovery was that the bifunctional enzyme AAC(6′)-Ib-cr was able to confer resistance to aminoglycosides and fluoroquinolones (Robicsek et al., 2006a). Recently, Maurice et al. (2008) have shown that substitutions W92R and D169Y in the AAC(6′)-Ib sequence are responsible for increasing its spectrum of action to quinolones. This enzyme is a good example of the surprising capacity for adaptive evolution of antibiotic-resistance genes.

We should have learned the lesson about the capacities of bacteria to develop antibiotic-resistance mechanisms when β-lactam plus β-lactamase-inhibitor antibiotic combinations and broad-spectrum cephalosporins were discovered in the

1980s. In those days, the original idea that antibiotic-resistant Gram-negative bacteria would be unable to develop resistance to β-lactam plus β-lactamase-inhibitor combinations was accepted by the scientific community (Medeiros, 1997). This idea arose because mutant β-lactamases able to confer resistance to broad-spectrum cephalosporins (ESBL) proved to be hypersusceptible to β-lactamase inhibitors, and vice versa. This mutational **pleiotropic antagonism** was confirmed in several in vitro experiments and, indeed, the combinations of mutations leading to resistance to both β-lactam plus β-lactamase inhibitor were not observed in nature. However, in 2003 a laboratory mutant of ROB-1 β-lactamase harboring changes S130G, involved in inhibitor resistance, and R164W and A237T, involved in ESBL resistance, was obtained, the host bacteria being resistant to both amoxicillin-clavulanate and ceftazidime (Galán et al., 2003). Later, in 2004, a new β-lactamase CMT (a complex mutant TEM), which confers a high level of resistance to ceftazidime combined with a reduced susceptibility to amoxicillin-clavulanate, was reported in a clinical isolate (Poirel et al., 2004).

12.4 Limitations to Adaptation and the Cost of Resistance

12.4.1 The Genetics of Adaptation

Genetic variability in a population does not increase inevitably along time, since it is the result of factors acting in opposite directions: some processes introduce new variation in the populations while others remove it. Two main processes deplete variation from bacterial populations: selection and drift. By increasing the proportion of cells that carry particular high-fitness variants, selection may transitorily reduce genetic variability in populations, while the effect of drift is continuous and affects equally all variants in the population, regardless of their effect on fitness.

Fitness is a relative measure of the contribution of an organism to the next generation. In its simplest formulation, fitness is an individual property but it can be, and usually is, extended to groups of organisms that share some common features such as belonging to the same lineage, sharing a specific mutation, or displaying a given phenotype. Despite its simplicity, fitness can be a very elusive concept. In the context of antibiotic resistance, fitness can be defined as the relative capacity of bacteria to survive and reproduce within an infected individual and to spread to infect others. The epidemiological component of this definition emphasizes the need for considering all the meaningful biological levels at which fitness can be analyzed. A very successful variant which can resist an antibiotic will be of very little relevance if it fails to be transmitted to other individuals. Both fitness components, intrahost and interhost, are usually correlated but this is not necessarily so. Evaluation of intrahost components can be approached using in vitro systems but the transmission fitness is only possible from epidemiological observations.

Fitness is not a fixed property of individuals or groups: it is contextual and it can change dramatically when the environment or the genetic background is

altered. This is readily exemplified when we talk about the cost of resistance. This is the reduction of the fitness of antibiotic-resistant bacteria in the absence of the drug. In the presence of that particular antibiotic, and occasionally in that of others as well, the increase in fitness associated to survival, reproduction, and transmission reveals the environmental dependence of the concept. Similarly, compensatory mutations can alter the fitness value of a certain resistance mutation by modifying the genetic context in which they are expressed. Naturally, both genetic and environmental changes can interact in synergistic or antagonistic ways, making more difficult the prediction of the phenotypic value under particular combinations of the two components.

Fitness is a relative measure of reproductive success and is usually evaluated as the difference between the population growth rate of a reference and the target strain (Elena and Lenski, 2003). Nevertheless, other measures of fitness are more useful for the study of antibiotic-resistant bacteria at the within-population level. For instance, survival under a given concentration of a drug is, at the individual level, a binary variable, but the proportion of individuals from a given population surviving under such conditions is a quantitative value. This allows us to rank strains or genotypes according to their fitness. Similarly, a given population may be resistant to higher concentrations of an antibiotic or the necessary concentration of this to inhibit completely bacterial growth (MIC value) can be higher. The values of these variables are often taken as indirect measures of fitness and they usually correlate with increased risk or potential harm in the clinical practice. A higher dose of an antibiotic may have serious side effects or may not be easily tolerated by some patients, thus posing at higher risk their survival from an infection.

At the population level, bacterial fitness is measured through R_0, the basic reproductive number. This is defined as the number of secondarily infected hosts from any infected individual. For any infection to persist in a population its R_0 must necessarily be larger than 1, although temporary persistence is possible even if $R_0 < 1$. The value of R_0 is computed in a population with a large proportion of susceptible hosts and, in consequence, R_0 cannot be constant in a real population, where it will decrease through time.

Genetic drift is the result of the sampling process that occurs in every population in which the total number of individuals is limited. This limit (which is equivalent to the infection dose in this context) can be very high (millions or billions, in the case of bacterial populations) or very low, as when an individual is infected by a single bacterial cell, as in some tuberculosis (TB) infections. In the former case, the reduction in genetic variability is almost imperceptible and it is easily compensated by the continuous generation of new genetic variation. On the contrary, extreme reductions in population size, especially during the transmission from one infected host to a new one result in a drastic elimination of genetic variability after which only a few of the initially present variants are represented in the newly established population. In this case, the variants that originate the new population are drawn at random from those initially present and the particular variants

transmitted are not necessarily associated with increased fitness: they can be more, equal, or less fit than those of the average population they derive from.

Although usually overlooked, if not ignored, in the study of genetic variation in microorganisms, the neutral theory of molecular evolution (Kimura, 1968; Kimura, 1983) sustains that most variation found at the molecular level does not have an impact on fitness and, as a consequence, is neutral in terms of natural selection. The original proposal was subsequently expanded (Ohta and Kimura, 1971; Ohta, 1992) by incorporating the evolutionary consequences of slightly deleterious mutations whose fate does not depend exclusively on the relative reduction in fitness they produce, but on the size of the population where they arise. Stochastic processes, usually associated with genetic drift, will dominate the fate of these mutations if effective population size is lower than the reciprocal of the corresponding selection coefficients. When population sizes or selection coefficients are larger and the above inequality no longer holds, then deterministic processes will dominate and selection will be the main evolutionary force in the population. Given the large population sizes associated with bacteria, it is usually considered that genetic drift is not as important as selection in determining evolutionary change in bacterial populations. But this is not the case during transmission or during chronic infection. Effective sizes for parasitic or pathogenic bacterial populations have been estimated to be much lower than free-living bacteria (Hughes, 2005). This implies that stochastic factors may have an important role in the evolution of bacterial pathogens at this level. One additional, often overlooked, aspect of the quasi-neutral theory is that it also applies to slightly favorable mutations. While some mutations may confer increased fitness, their dynamics (stochastic or deterministic) will be determined by the relationship between the effective population size and the selection coefficient: a slightly advantageous mutation may easily disappear from a small population while it will likely increase in frequency in a large one.

The interplay between selection and drift can have consequences and leave imprints at different levels. The study of the evolution of CTX-M β-lactamases toward higher MIC values for cefotaxime and ceftazidime (Novais et al., 2010) demonstrates that some critical steps in some of the evolutionary trajectories revealed in the analysis were only possible if drift had played an important role, since the fitness of a necessary new genotype in a pathway was lower than that of the preceding variant. Apart from invoking evolution in alternative environments (with different fitness landscapes than those considered), this is only possible by the action of stochastic factors, among which drift is the major player. At a different level, reduced population sizes in *M. tuberculosis* may explain the higher relative rates of non-synonymous substitutions in their genes when compared with other free-living bacteria (Comas et al., 2010). In consequence, although selection may be the dominant factor in the evolution of bacterial populations, explaining almost perfectly the observed dynamics of antibiotic resistance in the presence of the selective drug, other evolutionary processes cannot be dismissed completely as irrelevant. Since these dynamics do not depend on fitness advantages, it is not necessary to invoke a cost of adaptation in every case and, most especially, in the absence of antibiotic.

12.4.2 From Genotype to Phenotype: The Many Ways Toward Fitness Compensation

While it is true that there are examples of drug-resistance mutations with no associated fitness cost, it is clear from their usually low frequencies that a fitness cost in the absence of the drug tends to be associated with resistance. This observation has led some researchers to argue that removal of antibiotics will leave room for drug-susceptible strains and that these will outcompete those harboring drug-resistance mutations (Austin et al., 1999). Although the strategy of drug removal seems to have some success in particular settings (Seppala et al., 1997; Guillemot et al., 2005) it is clear that other factors apart from the total levels of drug use influence the frequency of drug resistance. One of these factors, as shown by several clinical, experimental, and epidemiological data, indicates that compensation of fitness cost occurs, so that the advantage of drug-susceptible strains in an antibiotic-free environment is reduced or even disappears (Andersson and Hughes, 2010). As discussed previously, the fitness cost can be ameliorated through reversion, which is a very unlikely process, particularly for chromosomal mutations where exactly the same codon has to change to the wild-type allele (Levin et al., 2000). It is more likely that, in the absence of the antibiotic, the low-fit drug-resistant strains either become extinct or find ways of recovering fitness while keeping a drug-resistant phenotype. This process is usually known as compensation. Compensation is much more likely than reversion because there are usually many more loci that potentially can restore, at least partially, fitness costs. These loci can be in the same gene harboring the drug-resistance (intragenic) mutation, in other genes that somewhat interact with the drug-resistance mutated gene (intergenic), in plasmids or in another chromosome, depending on the mechanisms giving more chances to compensate than to revert a drug-susceptible phenotype (Maisnier-Patin and Andersson, 2004).

Mechanisms leading to compensation of drug resistance can be grouped in three categories: (i) those based on chromosomal compensatory mutations, (ii) those based on some kind of regulation alteration of the expression, and (iii) those based on the so-called bypass mechanisms. Chromosomal mutations leading to compensation represent the case in which fitness loss is compensated by a second, or more, mutations. These mutations can occur either in the same protein affected by the drug-resistance gene (intragenic mutation) or in other proteins that interact with it (intergenic mutations). But the complexity of compensation mutational pathways can go far beyond the accumulation of one or two mutations. Marcusson et al. (2009) showed how in isogenic, lab-constructed strains of *E. coli* resistant to fluoroquinolones, sometimes higher fitness effects are only attainable when four or five mutations are combined in the same strain and always depend on the loci mutated. They even showed how in the absence of the drug lower susceptibility can be achieved as a by-product of mutations increasing fitness. Interaction between mutations can occur also among drug-resistance mutations for different antibiotics, sometimes leading to a higher (positive) or lower (negative) fitness than the mere sum of their individual effects, a phenomenon usually known as epistasis.

Epistasis, when positive, can explain why the frequency of high-cost drug-resistant strains in clinical settings is higher than expected. A clear example of these epistatic interactions is shown by Trindade et al. (2009). They introduced mutations to different drugs in isogenic strains, thus creating multidrug-resistant strains and focused on combinations of drug-resistant mutations to rifampicin (*rpoB* gene), nalidixic acid (*gyrA*), and streptomycin (*rpsL*). They found that several combinations of these mutations led to fitter than expected mutants. Furthermore, these mutations were not gene- but allele-specific, and therefore epistasis and compensation depended on combinations of particular codon changes. In some cases, the double mutants not only were fitter than expected from their individual mutant's fitness but also were fitter than at least one of the two individual mutants. This phenomenon is called sign epistasis and means that there is not only amelioration of the fitness cost between drug-resistant mutations (positive epistasis) but also compensation leading to partial restoration of fitness. It is interesting that combinations of *gyrA*, *rpsL*, and *rpoB* drug-resistance mutations have been shown to be present in different bacterial backgrounds, which suggests that epistasis among drug-resistance mutations can be present in many pathogens. In fact, multidrug clinical resistant strains of *M. tuberculosis* have been reported to have higher fitness than their rifampicin-susceptible counterparts, thus indicating that compensation during treatment and/or epistatic effects between different drug-resistance mutations ameliorate, or even revert, the fitness cost of individual changes (Gagneux et al., 2006a).

Another way to compensate for the fitness cost of drug-resistance mutations is at the level of the expression of a protein (Maisnier-Patin and Andersson, 2004). There are examples of upregulation, through mutation, of a gene to counteract the negative effects on the expression of drug-resistance mutations, processes usually known as bypass mechanisms. The most typical example is that of KatG and the upregulation of *ahpC* in *M. tuberculosis* (Sherman et al., 1996). Isoniazid is a prodrug and it needs the catalase-peroxidase activity of KatG to become active. Mutations in KatG confer isoniazid drug resistance. A whole spectrum of mutations altering KatG function have been described (Ando et al., 2010) and because the gene has an important role in the bacterial response to oxidative stress, it has been assumed that all of them have an associated fitness cost. It has been reported that upregulation of the *ahpC* gene due to a mutation in its promoter can partially compensate for the loss of activity of KatG. Although *ahpC* and *katG* remain as the best example of upregulation to compensate fitness loss, it is worth mentioning that there are conflicting reports about the occurrence of the promoter mutation of *ahpC* in nonresistant strains (Borrell and Gagneux, 2009).

Another common way to increase the expression of a particular product is by gene duplication and amplification (GDA) (Sandegren and Andersson, 2009). GDA is a common mechanism to confer resistance to many antibiotics. However, it has been shown to be also a way of compensating for the fitness loss associated to resistance. GDA as a compensatory mechanism has been demonstrated more clearly in experimental evolution tests with *Salmonella enterica* (Nilsson et al., 2006). Tandem duplications of the *metZ* and *metW* genes compensate for the loss

of methionyl-tRNA formyl-transferase by increasing levels of the non-formylated tRNA inhibitor, the one used by eukaryotes for translation initiation.

12.4.3 Beyond Model Organisms: Epidemiological and Experimental Fitness Cost *in* M. tuberculosis

Experimental evolution with model microorganisms has been a successful approach to test evolutionary hypotheses. These experiments allow studying evolution in real time, producing accurate measures of key parameters like fitness, generation times, population sizes, or mutation rates (Elena and Lenski, 2003). As we have seen above, drug resistance can be approached within an evolutionary framework given that antibiotics are the main evolutionary pressure a microorganism can face jointly with the host's immune system. However, many studies on antibiotic resistance have been done with organisms with limited public health impact like nonpathogenic laboratory strains of *E. coli* or *Pseudomonas* spp. These works have given insights on how microorganisms cope with antibiotics mainly because model organisms are easier to work with than clinical strains of pathogens, which usually have longer generation times and are more difficult to culture.

A paradigmatic, or even extreme, case in this respect is *M. tuberculosis*, the causative agent of TB, with a colony-forming time of 3−4 weeks and which requires working in BSL3 facilities. This is why alternative model organisms, like *M. smegmatis*, are frequently used to test hypotheses in TB research. However, *M. smegmatis*, which is a fast-growing nonpathogenic mycobacteria, has a genome size much larger than *M. tuberculosis* and many phenotypic differences, therefore caution must be taken when trying to establish parallelisms with *M. tuberculosis* (Barry, 2001; Reyrat and Kahn, 2001). Experimental work on drug resistance with *M. tuberculosis* has been successfully completed, corroborating many conclusions drawn from model organisms and justifying a constant feedback between model organism and real pathogens. A clear example is the case of the evolution of drug resistance against rifampicin. Rifampicin targets the β unit of the DNA-dependent RNA polymerase of microorganisms (encoded by the *rpoB* gene) by competing for the union to DNA and inhibiting RNA synthesis (Campbell et al., 2001). Therefore, it is a wide-spectrum antibiotic as it affects, with different efficiencies, many bacteria. Early work with *E. coli* (Ezekiel and Hutchins, 1968) and other bacteria identified homologous positions mutated in drug-resistant strains, both in experimental and clinical settings, something expected given the high conservation of the *rpoB* gene among bacteria. A screening of gene mutations in *rpoB* from clinical strains of *M. tuberculosis* was also able to identify many of them, as well as other mutations (O'Sullivan et al., 2005). However, these mutations varied in frequency, suggesting a possible difference in the degree of antibiotic resistance conferred and/or the fitness associated to them. Experimental evolution of two different lineages of *M. tuberculosis* revealed the existence of two main factors affecting drug-resistance fitness cost in this species: the genetic background of the strain and specific codon mutations (Gagneux et al., 2006b). Different codon

mutations were found to have different fitness costs. Furthermore, these fitness costs varied among two lineages of *M. tuberculosis*, although in both cases the change, S531L, was the one associated with less fitness reduction. Reinforcing these results, mutations with lower fitness costs were found to be the most frequent among clinical strains, suggesting a correlation between in vitro and epidemiological fitness cost. Finally, the fitness of pair isolates of rifampicin-susceptible (RIFs) and rifampicin-resistant (RIFr) strains coming from 10 different patients who converted to drug resistance during treatment were screened, showing not only comparable results to the experimental findings but also cases in which the fitness of the drug-resistant strain was higher than that of the drug-susceptible counterpart, a result that was explained by the occurrence of compensatory mutations and/or by epistatic effects of drug-resistance mutations, as we have seen earlier.

12.5 Can the Evolution of Antibiotic Resistance be Predicted?

Conventional scientific wisdom dictates that evolution is a process that is sensitive to many unforeseeable events and influences and, therefore, is essentially unpredictable. On the other hand, considering the tremendous amounts of recent knowledge about bacterial genetics and genomics, population genetics and ecology of bacterial organisms, and their subcellular elements involved in HGT, we should eventually face the possibility of predicting the evolution of bacterial populations and traits (Martínez et al., 2007). The importance of such type of approach is self-evident in the case of the evolution of antibiotic resistance and bacterial−host interactions, including infections. The prediction of bacterial evolution could provide similar clues as weather prediction, with higher probabilities of success in the closer and more local frames. Indeed there is a *local* evolutionary biology based on local selective constraints that shape the possible local trajectories, even though in our global world, some of these locally originated trends might result in global influences. In the case of adaptive functions (as antibiotic-resistance genes in pathogenic bacteria) some of the elements whose knowledge is critical for predicting evolutionary trajectories are: (i) the origin and function of these genes in the source environmental bacterial organisms; (ii) their ability to be captured (mobilized) by different genetic platforms and to integrate in particular MGEs; (iii) the ability of these MGEs to be selected, transferred, and spread among bacterial populations; (iv) the probability of intrahost mutational variation and recombination; (v) the probability of recombinatorial events among these and other mobile elements, with consequences in selectable properties and bacterial host ranges, (vi) the original and resulting fitness of the bacterial clones in which the new functions are hosted, including their colonization power and capacity to spread in an epidemic form; (vii) the results of the interactions between these bacterial hosts and the microbial environments in which they are inserted; and (viii) the selective events, such as the patterns of local antibiotic consumption or industrial pollution and, in general, the

structure of the environment that might influence the success of particular genetic configurations in which the adaptive genes are hosted. Dealing simultaneously with all these sources of evolutionary variation is certainly a challenge at present.

Such a type of complex structure has evolved along all hierarchical levels of biology, creating specific "Chinese-boxes" or "Russian-dolls" patterns of stable (preferential) combinations, for instance, encompassing bacterial species, phylogenetic subspecific groups, clones, plasmids, transposons, insertion sequences, and genes encoding adaptive traits. Assuming a relatively high frequency of combinatorial events, the existing transhierarchical combinations are probably the result on the local availability of the different elements (pieces) in particular locations (local biology), the local advantage provided by particular combinations, and also the biological cost in fitness of some of them. More research is needed to draw the interactive pattern of biological pieces in particular environments (grammar of affinities). Such a complex framework required for predicting evolutionary trajectories will be analyzed (and integrated) by considering heuristic techniques for the understanding of multilevel selection. The application of new methods, based on covariance, and contextual analysis, for instance using Price's equation (Price, 1970), should open an entirely new synthetic way of approaching the complexity of the living world.

12.6 Conclusions and Perspectives

In the absence of new antibiotics, most efforts have focused in protecting the few current ones that maintain activity, trying to reduce their strong selective effects by reducing antibiotic consumption in animals and humans while maintaining their efficiency. In a number of countries this collective policy has proven insufficient. It has been proposed that the control of antibiotic exposure should be considered by society as an individual-based attitude to reduce individual risks, using similar approaches to those for controlling tobacco-associated diseases, hypercholesterolemia, or hypertension (Baquero, 2007). Reductions in the host-to-host transmission of resistant organisms through innovative approaches trying to influence the ecology and evolution of resistant organisms might represent alternative ways to limit the spread of antibiotic resistance in the microbiosphere. In this respect, the possibility of applying in the future eco-evo drugs—drugs acting *not* to cure the individual patient but to "cure" specific environments from antibiotic resistance, and to prevent or weaken the evolutionary possibilities (the evolvability) of the biological elements involved in it—should be considered. In other words, this strategy proposes to combat (decontaminate, de-evolve) resistance not in infected patients, but rather in the whole population, including infected and noninfected people alike, as it occurs in hospitals, nurseries, elderly facilities, etc. By extension, other environments that can be successfully treated are farms, fish factories, or sewage facilities. Indeed, the notion of "ill environment" should be increasingly encouraged, and medical-like approaches might be increasingly applied to prevent and cure biologically altered environments (Baquero, 2009a).

The targets of these future drugs, some of them in early development, are not only resistant, "high-risk" clones but also the interbacterial transmissibility, the maintenance of bacterial plasmids and integrative-conjugative elements carrying resistance, the ability of transposons and integrons to move between genomes, or the mechanisms of bacterial adaptation to antibiotic stress, including control of mutation and recombination rates.

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Glossary

Founder effect the random change in genetic composition of a population due to a extreme reduction in its size during a colonization or infection episode.

Genetic drift the random change in the genetic composition of a population due to its finite size. Every population experiences genetic drift but its effects, a reduction in genetic variation eventually leading to fixation of a variant, are more intense, both in magnitude and speed, the smaller its population size.

Mutator strains bacterial strains with an increased mutation rate usually due to a defective mismatch-repair system.

Pleiotropic antagonism the effect of a gene on two different traits with opposite consequences on fitness.

Resistome the set of antibiotic-resistance genes or proteins found in a given environment.

List of Abbreviations

ESBL extended-spectrum β-lactamases
GDA gene duplication and amplification
HGT horizontal gene transfer
MDR multidrug resistance
MGE mobile genetic element
MIC minimal inhibitory concentration
MRSA methicillin-resistant *Staphylococcus aureus*
$\boldsymbol{R_0}$ basic reproductive number
RIFr rifampicin-resistant
RIFs rifampicin-susceptible
TB tuberculosis

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