Evolutionary Pathways and Trajectories in Antibiotic Resistance

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SUMMARY …… 12

INTRODUCTION…… 13

RESISTANT BACTERIA AND RESISTANCE GENES……15

Resistant Bacteria and Unsusceptible Bacteria…….18

The Antibiotic Resistome…….19

What is a Resistance Gene and How Does it Emerge?.....22

Emergence…… 22

The infinite universe of pre-resistance bacterial functions ……...23

The limits of the operational definition of resistance gene……... 26

Intrinsic Resistance Genes as Resilience genes……... 27

EVOLUTION: UNITS, TOPOLOGIES AND TRAJECTORIES…… 29

What does Evolution Mean when Applied to Antibiotic Resistance?..... 29

The Units of Evolution: Evolutionary Individuals…… 31

Evolutionary Topology of Antibiotic Resistance Trajectories ……... 33

Evolutionary Trajectories Interactions …… 35

The Question of Causality in Evolutionary Trajectories…… 36

Are there Random Trajectories? Stochastic "Drift" Evolution…… 37

Dispersal and spatial structuration as sources of drift…… 39

Fragmentation of infective populations, drift, and resistance…… 40

Drift, draft, and trajectories…… 41

Genealogical, Across-Network, and Spinning Trajectories……... 43

Diversifying and Unifying Evolution in Antibiotic Resistance…… 45

STEPS ALONG PATHWAYS: SOURCES AND FREQUENCIES OF VARIATION …… 48

Phenotypic Variation: Bet-Hedging Adaptive Strategies…… 48

Mutations Leading to Antibiotic Resistance …… 51

Mutation rates ……. 51

Mutation per species, gene, and day in a single host…… 52

Hypermutation in the evolution of antibiotic resistance……. 54

Mutational events by insertion ……. 56

Polyploidy and Gene Amplification: from Adaptation to Neo-Functionalization...57

Horizontal Gene Transfer…… 60

The genes subjected to horizontal gene transfer…… 60

Gene capture by transposable elements…… 62

Genomes in turmoil: gene acquisition, gene loss…… 62

Transferable resistance, recombination, and evolution…….. 63

DRIVERS OF VARIATION AND SELECTION SHAPING TRAJECTORIES UNDER ANTIBIOTIC EXPOSURE …… 64

Stress and Antibiotics as Drivers of Genetic Variation…… 64

Antibiotics as Drivers of Populational Variation …… 65

Selection for Resistant Non-Inheritable Phenotypes…… 67

Evolution of inducibility of antibiotic resistance mechanisms …… 67

Selection of persistence and the evolution of antibiotic resistance …… 72

Selection of Antibiotic Resistance …… 74

Antibiotic selective concentration gradients in time and space: concentration-dependent selection and multivariate landscapes……. 74

The "wicked problem" of modern antibiotic resistance and the history of antimicrobial selective events …… 78

Genetic capitalism: resistance traits, global fitness, and evolvability tools... 81

Pharmacodynamics and selection of antibiotic resistance…… 82

Strategies of antibiotic use and evolution of resistance; collateral sensitivity, collateral damage…….. 82

Resistance dynamics in the presence of diverse antimicrobials and antiresistance strategies…… 84

THE ECOLOGY AND TOPOLOGY OF EVOLUTIONARY TRAJECTORIES OF ANTIBIOTIC RESISTANCE …… 85

Trajectories and Fitness Landscapes of Antibiotic Resistance…… 85

Trajectories and Flows in Free-Energy Fitness Landscapes……... 87

Evolutionary Trajectories in Crumpled Landscapes…….. 88

Genotype by Environment Interactions: Environmental Merging and Coalescence of Microbiotas…….. 90

The Role of Environmental Heterogeneity ……... 91

Ecologically Cohesive Populations and Genetic Exchange Communities ……. 94

Antibiotic Resistance in Minority Populations…… 95

Host-Environment Equilibrium as an Evolutionary Constraint: Evolutionarily Stable Strategies …… 96

The Eco-Evolutionary Spaces of Gene Variation: Chromosomal Genes versus Mobile Genetic Element Genes …… 97

EVOLUTIONARY TRAJECTORIES OF ANTIBIOTIC RESISTANCE GENES. …… 98

The Gene Space of Variation……98

The gene mutational space ……... 98

Mutational cost and compensation: mutational robustness…… 101

Antibiotic resistance mutations: fitness costs …… 102

Antibiotic resistance mutations: compensatory evolution……... 104

Mutations competition, cooperation, and founder effects …… 105

Epigenetic epistasis shaping trajectories…… 106

Epistasis and hidden genetic variation…… 109

Mutational Paths in Genes Involved in Antibiotic Resistance…… 110

Mutational paths in target-resistance evolution ……. 110

Mutational paths in variant penicillin-binding protein mediated resistance. 111

Mutational paths in variant DNA topoisomerases …… 113

Mutational paths and target gene conversion……... 115

Mutational paths in evolution of detoxifying enzymes ……. 116

Inactivating enzymes: the case of beta-lactamases ……. 117

The case of aminoglycoside-inactivating enzymes…… 124

Mutational paths in efflux pumps ……. 125

Evolutionary trajectories of gene complexes……... 126

Costs and Benefits of the Acquisition of Foreign Antibiotic Resistance genes and Functions: the Question of Orthogonality…… 128

EVOLUTIONARY TRAJECTORIES OF MOBILE GENETIC ELEMENTS HARBORING RESISTANCE GENES …… 129

Ecology and Evolution of Mobile Genetic Elements ……... 130

Plasmids ……. 130

Transposable elements …… 135

Insertion sequences and insertion sequence derivatives …… 136

The Tn*3* superfamily …… 141

The Tn*7* superfamily ……. 142

Tn*5053*/Tn*402*-like transposons …… 143

Tn*552*-like elements …… 144

Non-autonomous transposable elements …… 144

Genomic islands …… 145

Bacteriophages and phage-related particles …… 146

Flow of Mobile Genetic Elements and Antimicrobial Resistance Genes …… 149

The environmental context of antibiotic resistance gene flow ……... 149

Gene flow and DNA uptake proficiency…… 152

Gene flow and conjugation proficiency…….. 153

Gene flow and the acceptability to foreign genes: defence systems…… 153

Barriers between prokaryotic groups and antibiotic resistance gene flow.. 154

Barriers determined by interactions between mobile genetic elements…….156

Mobile genetic elements dispersal within species ……157

Intracellular dynamics of mobile genetic elements…… 159

The intracellular "evolution laboratory" for antibiotic resistance…….. 161

The Ecogenetics of Antibiotic Resistance Transfer and Maintenance: Antibiotic Resistance Genes in the Accessory Genome…… 161

Trajectories of accessory genome genes in Gammaproteobacteria……... 162

Trajectories of accessory genome genes in Firmicutes……... 163

Evolutionary Kin Hindrances and Shortcuts: the Role of Relatedness…… 163

Facilitated recombination between gene families…….. 164

Facilitated gene transfer among relatives: species and clones…… 165

The eco-evolutionary advantages of relatedness: kin selection…… 165

EVOLUTIONARY TRAJECTORIES OF ANTIBIOTIC-RESISTANT CLONES AND SPECIES ……... 166

Evolutionary Dynamics of Resistant Clones ……... 169

The basic theoretical background: Red Queen, Stationary, and microbiota-on-a-leash models…… 169

Clonal fluctuations and evolutionary rescue…… .171

The structure of clonal fluctuations…… 173

New hosts invasion-driven genetic variation ……... 173

Intra-clonal diversification within hosts…… 174

Clonalization quashing, genetic and niche variation……. 174

Variation fostering cloud or bunch clonal selection……... 175

Clonal variation triggering community selection……... 175

Clonal diversity and antibiotic resistance……... 176

Clonal fluctuations and antimicrobial resistance……... 177

Traveling clonal waves and antibiotic resistance……... 178

Clonal mixtures, range expansion, spatial sorting, and evolution…… 179

High-Risk Species and High-Risk Clones…….. 180

Ecogenetics of High-Risk Species…….. 181

EVOLUTIONARY TRAJECTORIES OF ANTIBIOTIC-RESISTANT COMMUNITIES……. 181

The Trajectories of Microbial Communities…… 182

Resistance at the microbial community level ……. 184

Evolutionary trajectories in human microbial communities…… 185

Evolutionary trajectories and the microbiota community coalescence……. 187

Antimicrobials Influencing Ecology and Antibiotic Resistance in the Environment…… 187

Selection of Antibiotic Resistance by Nonchemotherapeutic antimicrobial inhibitors…… 190

Selection of Antibiotic Resistance by Water Decontamination Procedures…….191

The interplay of Antibiotic Resistance and Virulence……. 192

PREDICTING EVOLUTIONARY TRAJECTORIES……. 194

Experimental Evolutionary Trajectories……... 195

Long-term evolution experiments and historical contingency…….. 195

Evolution in empirical fitness landscapes …….. 198

Directed experimental evolution of antibiotic resistance……. 199

Experimental evolution of fitness costs of mutational variation or acquisition of resistance genes……..200

Directionality and Repeatability of evolutionary trajectories…….. 201

Complex Parametric Space, Chaotic Trajectories and the Strange Attractor. 203

Modeling Evolutionary Processes in Antibiotic Resistance……. 205

Mathematical models……... 205

Synthetic biology modelling evolutionary trajectories…… 206

Network analysis of evolutionary trajectories…… 207

Computational modeling of multilevel antibiotic resistance…… 209

ECO-EVOLUTIONARY INTERVENTIONS IN ANTIBIOTIC RESISTANCE. ……210

Targeting Emergence ……... 212

Targeting Transmission ……... 213

Targeting Restoration of Susceptibility……... 216

ANTIBIOTIC RESISTANCE AS A MODEL PROBLEM OF THE INFLUENCE OF ANTHROPOGENIC EFFECTS ON THE BIOSPHERE …….. 217

Anthropogenic Antimicrobial Agents on the Biosphere: Meta-selection of Antibiotic Resistance…… 217

Antibiotics, Antibiotic Resistance, and the Evolution of the Microbiosphere… 219

EVOLUTION OF ANTIBIOTIC RESISTANCE: A GLANCE FROM PHYSICAL SCIENCES. ……... 220

FINAL CODA: ABOUT THE INTELLIGIBILITY OF EVOLUTIONARY PATHWAYS AND TRAJECTORIES OF ANTIBIOTIC RESISTANCE…… 222

ACKNOWLEDGEMENTS …… 223

REFERENCES…….. 224

AUTHOR BIOGRAPHIES…….. 380

223 **SUMMARY**

224 Evolution is the hallmark of life. Descriptions of the evolution of microorganisms have
225 provided a wealth of information, but knowledge regarding "what happened" has
226 precluded a deeper understanding of "how" evolution has proceeded, as in the case of
227 antimicrobial resistance. The difficulty in answering the "how" question lies in the
228 multihierarchical dimensions of evolutionary processes, nested in complex networks,
229 encompassing all units of selection, from genes to communities and ecosystems. At the
230 simplest ontological level (as resistance genes), evolution proceeds by random (mutation
231 and drift) and directional (natural selection) processes; however, sequential pathways of
232 adaptive variation can occasionally be observed, and under fixed circumstances
233 (particular fitness landscapes), evolution is predictable. At the highest level (such as that
234 of plasmids, clones, species, microbiotas), the system's degrees of freedom increase
235 dramatically, related to the variable dispersal, fragmentation, relatedness or coalescence
236 of bacterial populations, depending on heterogeneous and changing niches and selective
237 gradients in complex environments. Evolutionary trajectories of antibiotic resistance find
238 their way in these moving, frequently random landscapes and become highly entropic and
239 therefore unpredictable. However, experimental, phylogenetic and ecogenetic analyses
240 reveal preferential frequented paths (highways) where antibiotic resistance flows and
241 propagates, allowing some understanding of evolutionary dynamics, modelling and
242 designing interventions. Studies on antibiotic resistance have an applied aspect in
243 improving individual health, one health and global health, as well as an academic value
244 for understanding evolution. Most importantly, they have a heuristic significance as a
245 model to reduce the negative influence of anthropogenic effects on the environment.

246

247 **KEYWORDS:** antibiotic resistance, evolutionary biology, trajectories, pathways.

INTRODUCTION

The evolution of antibiotic resistance has been frequently reviewed in recent decades (1–4). We are trying to offer here a different scope, not centered into the facts, but on the processes determining these facts. The main objective of this review is to examine the causal (deterministic) and stochastic processes that have shaped the evolution of antibiotic resistance. *Pathways* are sequences of changes that form chains in which each step facilitates the next, favoring, step by step, a significant increase in antibiotic resistance. However, antibiotic pathways explain only part of the *trajectories* of antibiotic resistance, which flow for numerous reasons in addition to antibiotic selection, in many cases taking tortuous paths determined by chance, involving unlinked and arbitrary events, or determined by selective events unrelated to antibiotic exposure. The classic theory is that evolution progresses in accordance with general biological laws along evolutionary pathways, describing trajectories for different variants of organisms and genotypes, to reach, step by step, significant antibiotic-resistant phenotypes.

In fact, the truth is less clear and directional, an inescapable consequence of the complexity of the entities that influence antibiotic resistance, which encompass various levels of biological hierarchies. Evolution cannot be traced along a single dimension (as a phylogenetic tree) but rather is the consequence of interactions in multiple dimensions, thereby resulting in multidimensional trajectories, following itineraries along a network rather than on a flat plane.

This review is less concerned about describing "*what* happened" in the history of resistance (the descriptive "stamp collecting" of facts, the classic activity of biology, in the ironic statement by Ernest Rutherford) than to approach "*how*", and more intent on covering the processes, mechanisms and reasons for the particular trajectories of antibiotic resistance. Bacterial organisms have a high degree of variability, and the

adaptive opportunities of their variants are fostered by the frequently immense population sizes and frequent exposure to changing environments. The "*how*" perspective might eventually identify "preferential" paths and trajectories in the evolution of antibiotic resistance, knowledge that is critical for preventing and controlling this significant public health problem. The face of evolutionary biology is changing from one that attempts to reconstruct and analyze the past to one that predicts future evolutionary processes, creating a "predictive theory of evolution" (5). The how-and-why approach, if directed at predictability, also needs a high degree of predictability, our logical way of judging, remembering, understanding, and communicating and thus is inevitably biased by the limits of our representation (6).

Ernst Mayr made a distinction between proximate and ultimate causes in biology (7–9)*;* using "proximate causation" to refer to the immediate factors (e.g., mutation, horizontal gene transfer) of processes and using "ultimate causation" with "final reasons" as the mechanisms causing the outcome (e.g., natural selection, evolution). The proximate causes constitute the chain of events that explain the final production of an effect, the "*how*"; which, in our case are the elements and processes creating the paths and trajectories that shape the current situation of antibiotic resistance. The ultimate causes are the reasons explaining the evolution of these paths and trajectories.

From an anthropogenic perspective, antibiotic resistance is a classical evolutionary process, based on a specific reaction (natural selection) by microbes to survive antibiotic exposure. However, this apparently ultimate cause might be "inhibited, prevented, reduced, facilitated, enabled, increased and otherwise affected by the presence of other causes. A cause is not the same as its manifestation". Antibiotic resistance occurs in an extremely complex and variable eco-biological system encompassing the whole planet, involving numerous other causes (10). Causality should therefore be clearly differentiated

from correlation alone (11). There are proximate and ultimate causes in antibiotic resistance; however, the existence of causes does not imply logic in the evolution of resistance, which is a blind process based fundamentally on chance (12). This review therefore focuses on the proximate causes, paths, and trajectories and only occasionally discusses the primary drivers of such processes. Studies on evolutionary paths and trajectories of antibiotic resistance are scattered throughout the scientific literature. We would like to offer a more integrative view. By increasing our knowledge about paths and trajectories, we might eventually predict relatively close trends in antibiotic resistance. The predictions of evolutionary paths and trajectories reviewed in this work resemble meteorological predictions, which also consider chance and necessity.

RESISTANT BACTERIA AND RESISTANCE GENES

From an anthropocentric, clinical standpoint, a bacterial organism is defined as antibiotic resistant when the chances of success when treating an infection produced by this organism with a specific antibiotic are low. Bacterial species can be intrinsically resistant to certain antibiotics (*European Committee on Antimicrobial Susceptibility Testing. 2016. EUCAST expert rules. Version 3.1. Intrinsic resistance and exceptional phenotypes tables.*); consequently, infections caused by these species should not be treated with these antibiotics. Other organisms, however, belong to bacterial species catalogued as susceptible to those antibiotics. When there is resistance in this case, it is related to the *acquired* ability of the originally *susceptible* bacterial organisms to survive and reproduce when exposed to antimicrobial agents. More simply, acquired resistance is a phenotype dependent on the modification of existing genes or on the acquisition of novel genes; the genes responsible for the resistance phenotype are the so-called “resistance genes.” In contrast to the situation with intrinsically resistant microorganisms, the risks of therapeutic failure are higher if only pathogen identification is performed. The actual

susceptibility to the various antibiotics typically administered for treating particular infections needs to be determined to implement the correct therapeutic procedure. The detection of resistance genes in genomes or metagenomes should be carefully evaluated to predict the risk of therapeutic failure and the dissemination of harmful resistance traits (13).

Over the last half century, there has been a broad consensus on the criteria for classifying bacteria as antibiotic susceptible or resistant. For clinical purposes, susceptibility signifies treatability, which is based on the toxicological, pharmacodynamic, and pharmacokinetic properties of the antibiotic in question and on the clinical information from clinical trials and the cumulative experience of antibiotic success in treating particular infections (14); however, a lack of therapeutic success might be unrelated to the resistance of the offending organism. For epidemiological purposes, a more "natural" method for defining susceptibility is based on recognizing that a particular bacteria belongs to the majority of susceptible wild-type populations of the species (13). A resistant bacterium is considered "untreatable" or "requiring a significantly higher amount of antibiotic to become inhibited than for most strains of the species". Resistance is frequently relative and can depend on the drug's pharmacokinetics and pharmacodynamics (PK/PD) (15). A worldwide effort to standardize criteria has led to the universal criteria for "resistance" (based on "breakpoints") for the various antibiotics (10). These breakpoints, which separate susceptible and resistant bacteria, are however mainly based on a single pharmacodynamic parameter: the antibiotic's minimum inhibitory concentration (MIC) under standard defined "*in-vitro*" conditions. The benefits of using the MIC include a standardized approach and the possibility of conducting comparative studies on the resistance rate among countries, but to a certain extent have hindered attempts to gain a more complete picture of the phenotypic differences between isolates exposed to

antimicrobial agents. In fact, bacterial organisms with identical MIC values might differ in the kinetics of antibiotic action (16). Breakpoint-based MICs are not available for a large majority of microorganism species, such as environmental bacteria that do not infect humans (17), or for several relevant antimicrobials, such as biocides, which are not employed for human therapy (18) except for body or tissue decontamination procedures (19–21).

The criterion for an abnormal MIC level (when compared with most strains of the species) can provide epidemiological cutoffs (ECOFFs) that define microorganisms with acquired resistance mechanisms as those that present MIC values above the upper limit of the normal distribution (wild-type population) in any given species or for any given compound, regardless of whether this information has clinical relevance (22–24). By using this approach, we can study bacteria and antimicrobial compounds without clinical relevance, as well as biocides, for which classical breakpoints have yet to be defined. The major drawback for this definition is that it requires analyzing a large number of independent isolates to obtain reliable information on the normal MIC distribution for a bacterial species/antimicrobial compound pair. The ECOFFs do not sufficiently account for the diversity of low-level resistance mechanisms in different intraspecific populations, which has been addressed in the resistant-population cutoff (RCOFF) approach (25).

The proposed *operational* definition for resistance (13) is based on the pairwise comparison of a parental (wild-type) strain with another derived strain either carrying an acquired putative resistance determinant or containing a mutation that alters its antibiotic susceptibility. If the wild-type parental strain is more susceptible than the derived strains, the acquired gene should be considered a “resistance gene” and the mutation a “resistance mutation”, irrespective of the resistance level achieved, which could help predict future trends in the emergence of resistance(26–28). The directed evolution of multiple genomic

loci has been proposed to improve such predictions (29). If the mutants obtained are more susceptible than the wild-type strain, the mutated genes probably correspond to those that contribute to the characteristic natural or intrinsic antibiotic susceptibility phenotype and, in this sense, are considered intrinsic resistance genes (30–32). The exact number of antibiotic resistance genes (ARGs) is unknown but extremely large; a list of 8000 sequences has been employed in gene-capture studies with the aim of characterizing the intestinal resistome (33). There is a long and continuously growing list of acquired (nonintrinsic) ARGs and their alleles (34) thanks to widespread whole genome sequencing technology, but this information is extremely biased by the overrepresentation of clinical and epidemic strains in databases.

Resistant Bacteria and Unsusceptible Bacteria

Based on the populational ECOFF definition of resistance, any microorganism that falls beyond the normal MIC distribution for a given bacterial species should be considered resistant. From a clinical standpoint, however, it is important to distinguish between resistant bacteria (those that have acquired a resistance phenotype) and unsusceptible microorganisms that were naturally antibiotic unsusceptible (intrinsically resistant) before anti-infective therapy was available. Any bacterial species is naturally unsusceptible to some antimicrobials (e.g., Gram-negative bacteria are intrinsically resistant to glycopeptides) but can, under antibiotic selective pressure, acquire resistance to those antibiotics to which they were naturally susceptible. For those antibiotics to which bacteria are known to be naturally unsusceptible, susceptibility tests are not needed. However, such tests are required to establish the right therapeutic procedures in the case of antibiotics for which bacteria are naturally susceptible but can acquire resistance.

Given this situation, most efforts to analyze antibiotic resistance have concentrated on acquired resistance, whereas the study of the elements making bacteria unsusceptible to these drugs has, until recently, received less attention. The recent interest arose from the study of the intrinsic resistome of bacterial pathogens, understood as the set of genes whose mutation increases a given bacterial species' antibiotic susceptibility (30, 31). The finding that several different mutations might increase antibiotic susceptibility (35–39), including to those antimicrobials to which the studied bacteria are resistant from a clinical standpoint, might enable the sensitization of previously unsusceptible organisms and increase the activity of antibiotics even in bacteria that are already considered susceptible (32, 40).

If an organism is considered susceptible when the antibiotic reaches the target at a sufficient concentration to inhibit the target's activity, there are two explanations for antibiotic insusceptibility: (1) the bacterium lacks the antibiotic target, or the antibiotic-target interaction is too weak to allow for the inhibition of the latter, in which case sensitization of the unsusceptible microorganism is not possible, which also occurs if the antibiotic requires an activation step (e.g., isoniazid, metronidazole) and the unsusceptible bacterium does not possess the enzyme responsible for this activation; and (41) although the antibiotic can recognize the target, its intracellular concentration is too low, which can be due to reduced permeability or activity of efflux pumps or to the action of housekeeping multidrug efflux pumps (42). This is the situation with many macrolides, which are not effectively accumulated by Gram-negative bacteria and cannot then inhibit protein synthesis in this group of microorganisms. This is the same situation with bacteria that carry housekeeping antibiotic inactivating enzymes.

The Antibiotic Resistome

The concept of the antibiotic "resistome" was proposed by G. Wright to describe the ensemble of genes (and their precursors in both pathogenic or nonpathogenic bacteria) present in a given habitat or bacteria and able to confer resistance to a certain antibiotic (43, 44). Several recent studies have explored the presence of ARGs (45–52) in various ecosystems with the aim of predicting the future emergence and spread of resistance(20, 27, 28, 53). According to functional genomic assays, any ecosystem contains its own ensemble of genes capable of conferring resistance in a heterologous bacterial host. Few of these genes have previously been detected as having been acquired through horizontal gene transfer (HGT) by human pathogens, and the overall structure of the resistomes is linked to their phylogeny (51) indicating that most resistance genes present in microbiomes belong to the intrinsic resistome. These findings agree with studies on the intrinsic resistome of bacterial pathogens, which show that up to 3% of the bacterial genome (100–200 genes per genome) might contribute to antibiotic resistance (35–39). Considering the number of different species present in any given habitat and the diversity of microbiomes in various environments (54, 55), there are likely millions of genes in nature capable of conferring resistance to antibiotics in a heterologous host.

In contrast, there are only a few hundred genes that have actually been acquired by human pathogens and constitute a risk for human health. As occurs with the TEM and OXA beta-lactamase families, they are occasionally alleles derived from the same gene (56). This misbalance between the number of genes able to be transferred and that confer resistance to human pathogens and the actual number of genes that have been acquired by such pathogens indicates that, despite their relevance for expanding our knowledge of the elements that have the ability to confer resistance, the predictive potential of these types of studies is low in comparison.

When attempting to predict antibiotic resistance, there are two types of systems that need to be considered from an ecological point of view. The first is formed by closed systems, defined as those that can be analyzed in full due to their limited complexity. An example of a closed system is a bacterial isolate, which can be sequenced, mutated, and subjected to experimental evolution. A number of strain or species can be analyzed in detail due to their limited complexity, which allows determining the genes that contribute to antibiotic resistance (either acquired or intrinsic) can be achieved using current tools, which supports the feasibility of tracking the resistome for key relevant isolates. This task is more difficult for bacterial species presenting small core genomes and large pangenomes (such as *Escherichia coli*) than for species such as *Pseudomonas aeruginosa*, which present large core genomes. The pangenome is the ensemble of all genes present in members of the species and consists of the core genome (including the genes found in all members of the species) and the accessory genome, genes that are present in only one or a certain proportion of the group members (57). When analyzing the pangenome of a species, the increase in the intrinsic resistome is expected to be proportionally incremental to the number of different isolates analyzed, which also applies for mutation-driven resistance. The exploration of mutant libraries and the implementation of evolution experiments under different conditions (58, 59) might help determine the universe of mutations capable of conferring antibiotic resistance, even for antimicrobials still under development.

The second category is formed by open systems, which primarily comprise ARGs acquired by HGT. We can determine the genes and the elements involved in their dissemination that currently contribute to resistance, but we cannot predict which gene will come next. For this type of element, the study of the hierarchical structure (60, 61) of the elements involved in the dissemination of resistance (e.g., genes, integrons,

transposons, plasmids, clones, species, hosts, ecosystems), together with an analysis on co-resistance, plasmid stability, and fitness costs could help establish the networks involved in the dissemination of resistance and are likely to predict the trends for the future spread of antibiotic resistance (28). Nevertheless, forehand knowledge of the first transfer event of the resistance gene from the original host to a pathogenic microorganism before this event occurs is not possible (62), an uncertainty that is the consequence of the aforementioned large number of potential resistance genes present in any ecosystem, which then constitute an open system that is composed of an overwhelming number of elements that are almost impossible to fully catalog within a reasonable time frame. In addition, this first transfer event has a large degree of serendipity, which impedes the use of deterministic approaches for predicting this emergence. Although the study of the antibiotic resistance mobilome, understood as the set of resistance genes present in mobile elements(44)(63), could help in the early detection of novel and potentially relevant resistance genes before they disseminate among bacterial pathogens (64). Determining which novel antibiotic resistance gene among those present in a given microbiota will transfer and constitute a problem for human health is likely beyond our abilities.

What is a Resistance Gene and How does it Emerge?

Emergence. The term "emergence" intuitively indicates the act of becoming known or coming into view (65) and refers to pieces (sequences, genes, replicons, populations) and patterns (the ordered, meaningful combinations of pieces influencing the natural engineering of antibiotic resistance) (60). The current meaning of emergence in evolutionary biology is highly influenced by the conceptual framework of systems biology (66, 67) and has been expanded to encompass various concepts and types of emergence (68, 69). A key issue in these concepts is that emergence requires observability, i.e., something might exist but only emerges if the emerging entity achieves

the abundance to reach the boundaries of visibility, which, in principle, implies growth as a prerequisite (69). In this age of advanced technologies, growth might become an increasingly less necessary condition, given the power of our analytical instruments and the criteria for identifying evolutionary individuals (see later, section 2.2) potentially enabling the recognition of the first bursts of emergent phenomena, such as in studies of ancient DNA focused on antibiotic resistance paleomicrobiology (70).

The infinite universe of preresistance bacterial functions. The clearest answer to the question "what is an antibiotic resistance gene?" is the evolutionary one (13). ARGs were present in the microbiosphere before the anthropogenic release of antimicrobials (49, 71), which probably explains the presence of ARGs in the metagenome of remote, pristine soil (72). Most ARGs were not born as resistance genes but as genes that encode the basic functions of cell machinery. There are, for example, the seemingly infinite variety and ubiquity in the bacterial world of modifying enzymes, such as acetyl-transferases, methylases, nucleotidyltransferases, esterases, phosphorylases, peptidases, thioltransferases, hydroxylases, glycosyltransferases, and oxidases. Modifying enzymes act in a diffuse manner on multiple targets, contributing to phenotypic versatility (73). These functions have the potential of reducing inhibitory activity or inactivating past, present, or future antibiotic substances, and antibiotic exposure has likely contributed to the evolution of these genes by forming efficient ARGs. The evolution of genes involved in metabolic pathways has probably followed a similar trend, such that current efficient enzymes are likely the result of the evolution of relatively inefficient small enzymes of broad specificity and the availability of suitable substrates, forming increasingly more substrate-specific enzymes (74, 75). The same pattern was probably followed in the case of antibiotic resistance, and significant resistance genes can be conceived of as "exaptations", in which a sequence coding for a particular function evolves to produce

another function required for novel adaptations (76, 77). In our view, however, exaptations maintain the functional core of the pristine trait.

In a universe of potential resistance mechanisms, everything depends on selective events. Expanding on the classic Baas-Becking hypothesis, “every gene is everywhere, but the environment selects” (78). The antibiotic might have a chance encounter with one of these pre-existing gene-encoded functions; perhaps this coincidentally provides a certain inactivation of the antibiotic compound. In this case, the bacterial organism expressing such a function (certainly with a purpose other than resistance) will increase in fitness in the presence of the antibiotic (i.e., it will be selected). For example, aminoglycoside acetyltransferases are part of the superfamily of Gcn5-related N-acetyltransferases sharing domains allowing use of acyl-CoAs to acylate different types of substrates. These aminoglycoside-resistance genes are also able to acetylate eukaryotic histones (79). If the exposure is frequent, the selected function should increasingly augment the genes’ ability to detoxify the antibiotic, closing in on an efficient antibiotic resistance gene. Sequences that code particular protein domains that are more common in the total pool of genomes appear to have a proportionally higher chance of being transferred (80, 81).

This process of emergence of resistance genes can be accelerated by combinatorial events involving the building up of complex (chimeric) proteins from sequences determining protein domains; i.e. protein sequences able to evolve and function independently. For instance, the metallo-beta-lactamase protein fold is a protein domain contained in class B beta-lactamases and in many other proteins unrelated to resistance, such as thioesterases, glyoxalases, and DNA-acquisition competence proteins (82). Synergies between genes involving mechanisms of resistance directed at the same group of antibiotics might evolve by the fusion of pre-existing genes, as in the case of the 2”-aminoglycoside phosphotransferase and 6'-aminoglycoside acetyltransferase “bifunctional enzyme” (83).

There are numerous bacterial genes whose function is still unknown, even in such well-known pathogens as *Escherichia coli* (35% of genes) (84). Advances in the functional determination of bacterial genes whose function has been considered unknown until recently has revealed a wealth of new candidate resistance genes in diverse microorganisms (85). Preresistance genes can be assumed by searching variant stochastic sequences of the canonical resistance genes, based on obtaining homologous proteins by applying a hidden Markov model (33, 86), or sequences with increased susceptibility phenotypes in transposon mutants (RB-TnSeq) of unknown function genes (85) or sequences predicted as involved in resistance by pairwise comparative 3D modelling with canonical resistance genes (87).

The possibility that antibiotic-resistance genes might also emerge as *de novo* genes, i.e., new genes derived from changes of the noncoding segments of the genome (88–90), is almost unexplored (91). However, synthetic proteins have been obtained from the noncoding DNA of *E. coli*, and a number of these pseudogene-derived proteins were predicted to be enzymes (92). Random sequences can also evolve rapidly into *de novo* functional promoters (93), eventually increasing ARG expression.

All these emergent evolutionary processes ultimately depend on antibiotic exposure. Given that antibiotics are natural compounds present in the environment, it is conceivable that the microbial populations coexisting with producers should have mechanisms to avoid the antibiotics' activity (94). Antibiotic producers must also have detoxification systems that serve to counteract the activity of the antimicrobials they produce. Although detoxification systems should not be considered as *bona fide* resistance genes given that they do not serve to resist a competitor, they still fall into the category of elements that

might have evolved to avoid the action of antimicrobials. In agreement with this, an earlier study suggested that the origin of resistance genes might be the antibiotic producers (95, 96). Indeed, producers present resistance genes belonging to the same structural and functional families as the ones currently acquired by bacterial pathogens. However, in the few cases in which the origin of resistance has been tracked, such a gene was not present in a producer, and it is difficult to believe that the gene was selected for conferring antibiotic resistance in its original host. A clear example of this situation is the quinolone resistance gene *qnrA*, now widespread in various plasmids (97). Genes belonging to this family are housekeeping elements present in the chromosomes of *Shewanella algae* and *Vibrio* species, which are not antibiotic producers (98). Quinolones are synthetic antibiotics, which makes it difficult to accept that *qnrA* evolved in nature for millions of years to overcome the action of this human-produced antimicrobial. Due to their widespread presence in species from aquatic environments, a basic physiological function could be suggested (99). The function of resistance is acquired just as the gene becomes decontextualized in a new host(14, 41, 100), when challenged with antibiotics in clinical settings and in wastewater polluted with residual fluoroquinolones (101). Bacteria that are antibiotic producers have resistance genes but probably currently play a minor role in generating clinical resistance (102).

The limits of the operational definition of resistance gene. From an operational perspective, a resistance gene produces resistance in a bacterial host, beyond its evolutionary and ecological prehistory. In this context, a resistance gene makes bacteria hypersusceptible upon the gene's inactivation and more resistant if it is expressed at a higher level than normal or when transferred to a new host (13). Using this definition, a number of regulators can be included in the category of resistance genes; however, resistance genes should be considered those whose expression is triggered by such a

595 regulator but that are not regulators themselves. Thus, even when using an operational
596 definition of resistance, manual curation is needed for interpreting the results of blind
597 high-throughput studies of antibiotic resistomes, implying that the number of potential
598 resistance genes largely exceeds the number of those that are homologous to classical
599 resistance elements, such as antibiotic inactivating enzymes and efflux pumps. Genes
600 involved in bacterial metabolism or target genes can provide resistance when expressed
601 in a heterologous host (24), despite the fact that they do not resemble classical resistance
602 determinants, as occurs with the donors of resistance, which are not confined to antibiotic
603 producers. Any bacterium that is ecologically connected with a bacterial pathogen can
604 therefore be the origin of a resistance determinant of potential health concern.

605 **Intrinsic Resistance Genes as Resilience Genes**

606 Resilience is the property of a system to return to a stable state following a perturbation.
607 During antibiotic exposure, the biodiversity of the microbiota is altered. An option for
608 regaining the original diversity is the reacquisition of the lost populations, typically by
609 food contamination, as occurs with animals when food is heavily contaminated by feces
610 (103). Even without transmission, however, the microbiota has the adaptive capacity to
611 fight against deep perturbations. Genes of the intrinsic resistome that provide antibiotic
612 resistance are not in a strict sense necessarily ARGs, understood as those that have been
613 recently (in evolutionary terms) acquired as the consequence of antibiotic use for treating
614 bacterial infections. Irrespective of the function these genes might have on their original
615 hosts, one of their possibly relevant functions in the recipient organism is conferring
616 resistance to antibiotics employed for therapy.

617 By maintaining their basic housekeeping functions, the genes of the intrinsic resistome
618 *de facto* protect their hosts from antibiotic exposure. For instance, AmpC beta-lactamases
619 from enteric gammaproteobacteria, which provide resistance to beta-lactam agents, have

evolved in mammalian gastrointestinal systems over millions of years, in which no beta-lactam producers have been reported. Chromosomally encoded “antibiotic resistance” efflux pumps are highly conserved and might have evolved via physiological functions and not due to antibiotic exposure (104–108). Given that these “intrinsic resistance genes” code for physiologic-ecologic functions, they are present in all (or most) isolates of a given species, generally contributing to some degree of insusceptibility.

In an antibiotic-polluted world, intrinsic resistance genes enable bacterial populations that harbor them to persist in the presence of antimicrobials, thereby contributing to selection over more susceptible organisms. Most such selection occurs without a previous mutation or acquisition of foreign genes. Intrinsic resistance genes, which are present and are maintained irrespective of the presence of antibiotics, can therefore be better considered as antibiotic *resilience* genes. Resilience refers to a system’s ability to recover from a disturbance (109). Thanks to intrinsic resistance, the resilience of many of the components of complex microbiotic systems (e.g., intestinal microbiota) is ensured when confronted with antibiotic exposure, but antibiotic resilience is a coincidental effect of their functions. In other words, the functional relevance of resilience genes is to ensure canalization of the microbiota in the presence of disturbing agents able to break the environmental integrity of the microbial system (87, 110). Environmental canalization is defined as the property of a biological system to maintain the normal standard phenotype despite environmental perturbations. Although most resilience genes belong to the core genome of bacterial cells, they can contribute to expressing antibiotic resistance only when their level of expression changes. Classical examples of this situation are chromosomally encoded antibiotic inactivating enzymes or efflux pumps, whose overexpression confers clinically relevant antibiotic resistance. In this case, the basis of

resistance are mutations at the regulatory elements of the resilience genes, not the presence of the genes themselves.

Distinguishing resilience genes within the overall resistance genes might aid the analysis of the risks associated with the presence of these genes in a microbiota (13), which are currently grouped together and ranked similarly. Resilience genes are "markers" of the normal microbiota, and variation in the content of resilience genes might influence the stability of bacterial communities (110). Concerning the evolution of antibiotic resistance, the most important effect of resilience genes and canalization is the preservation of an important part of the indigenous microbiota under antibiotic exposure, thereby limiting the selective effectiveness of drugs on antibiotic-resistant organisms.

If massive exposure to anthropogenic antibiotics has altered the effectiveness of resilience genes in improving the detoxification activity of commensal organisms, then the blurring of the distinction of resilient genes within resistance genes could be a key field of research that has been scarcely explored. However, this blurring occurs when widening the substrate spectrum of AmpC beta-lactamases (111, 112). The opposite phenomenon might also occur. Low-level intrinsic resistance is reduced in long-term laboratory experimental evolution assays in the absence of antibiotics, typically after 2000 generations in *E. coli* (113), which further supports the concept that intrinsic resistance genes are relevant elements for stabilizing bacterial populations in their natural habitats, yet they can be dispensable when bacteria face novel environments.

EVOLUTION: UNITS, TOPOLOGIES AND TRAJECTORIES

What does Evolution Mean when Applied to Antibiotic Resistance?

667 The term “evolution” originates from the Latin word “*evolutionem”* (to unroll as one
668 would a scroll book), thus providing a highly suggestive image of gain of information and
669 adaptation. The term was first employed in its modern form in 1832 by the geologist
670 Charles Lyell, who significantly influenced Charles Darwin’s in the conception of the
671 “Origin of Species by means of Natural Selection”, the founding text of evolutionary
672 biology (114). In its original meaning, *evolutionem* implies that what is currently visible
673 now is the present phase of a *continuum*; in biology terms, it means that present organisms
674 have direct ancestors and will have successors, in both cases hidden (past and future) as
675 in the scroll. This seminal metaphor applies identically for pages in a book or a compass
676 in a musical score. Essentially, what we perceive now can be explained by what came
677 before. What is of interest for this review is whether is if what we see now as
678 “observations of antibiotic resistance” has have been *determined* by preexisting biological
679 features, much as the content a page in a book is “determined” by the previous pages. As
680 previously noted, our interest is less “what happened” in the evolution of antibiotic
681 resistance, than “how” and, more obscurely “why” it occurred.

682 To study the “how and why” implies the possibility that the evolution of antibiotic
683 resistance can in fact be understood; in other words, whether the evolution of antibiotic
684 resistance can be predictable. The major difficulties in predicting antibiotic resistance are
685 related to i) the complexity of the biological and environmental components shaping
686 antibiotic resistance and ii) the influence of the randomness of biological and
687 environmental processes on the evolutionary uncertainty of resistance (115, 116).

688 What does “evolution” mean when applied to antibiotic resistance? Evolution is a basic
689 global phenomenon in biology, and bacterial organisms essentially evolve to *increase*
690 *their abundance* as much as possible, which eventually includes the development of
691 resistance to growth-inhibitory substances against competitors. The main objective of

692 evolution is to enable organisms (evolutionary individuals at large, see 2.2.) to survive
693 indefinitely. Achieving abundance and space helps ensure persistence over time (117).
694 There is no evolutionary success without persistence; the evolutionary arrow cannot be
695 broken. In the case of organisms that are strongly dependent on a fixed environment (e.g.,
696 intracellular bacteria, endosymbionts, phages in bacterial cells, and bacteria with
697 antibiotic-dependent growth), evolution is constrained and eventually will regress,
698 restoring the original adapted master copy. Purifying selection (removing non-
699 advantageous mutations) leads to genomic erosion mediated by small or large deletions
700 resulting from frequent DNA homopolymers (118). Thus, even if the evolutionary arrow
701 cannot be broken, evolution does not necessarily always progress forward, at least as
702 structural or networking advances.

703 Evolution is a stress-reducing process, where the engine driving it consists of the potential
704 difference between an organism's current fitness and the possibility for better fitness, to
705 thereby bring it more in balance with its environment. This difference can be expressed
706 as a difference in stress, with equilibrium generally being awarded with lower stress and
707 successful replication. Under the concept of "ultimate cause", antibiotics are thus stressful
708 agents for microorganisms; evolution works to minimize this stress by developing
709 antibiotic resistance mechanisms. Stress is fear of entropy and the loss of order and
710 integrity. A tempest of noise is frequently the immediate response to stress, fighting
711 entropy with noise in the hope of a creative solution. The problem lies in whether
712 exposure to successive stresses (and solutions) diverts the biology of the evolutionary
713 individual far from the first equilibrium point; i.e., if evolution is a diversifying force. As
714 we will discuss later in this review, there is a possible link between successive antibiotic
715 exposures, spread, clonal diversification, and entropic evolution.

716 **The Units of Evolution: Evolutionary Individuals**

The nature of units of evolution (the evolutionary individual) is critical for understanding antibiotic resistance processes and trajectories (119, 120) (figure 2). Trajectories of which kind of biological objects? There should be a network of paths associated with the evolution of different types of individuals, biogenic units (121) with growing information complexity, from molecules to organisms and communities. How can we approach the identification of evolutionary individuals, the biological units sequentially modified in time by natural selection? As a condensation of the concepts of Stephen J. Gould (122, 123), there are four minimal criteria to define an evolutionary individual: 1) reproduction, given that the individual is a replicator and biological evolution is a genealogical process; 2) inheritance, given that the informative attributes of the individual should be faithfully maintained in their progeny; 3) variation, given that a certain degree of variability in the progeny is needed to provide informative novelties in populations, and ultimately targets (traits) enabling natural selection to act; and 4) the ability to interact, that is, the ability to enter into the dynamics of individual-environment causal interactive relations, resulting in the selection of particular variants in the population that are the best fit for particular conditions or stressful changes. Reproduction, inheritance, variation, and interactive relations clearly occur from the lowest hierarchies, starting with genes. However, evolutionary individuals also encompass larger sequences (such as operons), cellular genomes, mobile genetic elements (MGEs) (such as phages, transposable units and plasmids), cells, clonal populations, species, multispecies assemblies, and holobionts (hosts and microbiota as single biological entities) (124–127) (Figure 1)

The key concept is that these evolutionary units are individuals that can evolve independently but are frequently embedded in each another, resulting in the integration of lower level replicators into high-level replicators. At each step, this integration constitutes a novel individual, with particular adaptive needs and possibilities for co-niche

742 construction (128, 129), which occurs asymmetrically, following hierarchy-selected
743 events. Therefore, the evolution of any unit at any level of the hierarchy might influence
744 the evolution of all others, both in a top-down and in a bottom-up dynamic, creating a
745 complex multidimensional landscape where the evolution of antibiotic resistance flows
746 along hierarchies. The most important issue is that the relationship among these units is
747 highly asymmetrical. Not every resistance gene is in every mobile element, not every
748 mobile element is in every bacterial clone or species, and not every bacterial species
749 belongs to every bacterial community or to every type of host. There are recognition codes
750 between evolutionary units; in fact, understanding evolutionary trajectories will depend
751 on deciphering these hypercodes (65, 124, 130, 131). These recognition codes, which give
752 rise to transhierarchical interactions, are the precondition for emergence of novel entities
753 (132).

754 **Evolutionary Topology of Antibiotic Resistance Trajectories**

755 Evolutionary trajectories of antibiotic resistance (a collection of phenotypes) occur within
756 a complex space of G-types (genotypes, genomotypes, and metagenomic types)
757 corresponding to the whole variety of evolutionary individuals. Each of these G-types has
758 a room of possible variation in space and time, eventually discontinued (punctuation),
759 irreversible, change-constrained, or able to progress in novel directions (innovation)
760 (133). The interactions among these spaces of variation essentially provide a virtual space
761 of *accessibility* distributions allowing the flow of evolutionary trajectories. The
762 accessibility of a phenotype is represented by genotype-phenotype maps (134), which
763 determine how phenotypes vary with genotypes.

764 An evolutionary trajectory can be viewed as a map from the time axis into the virtual
765 space of phenotypes that are accessible due to the existence of G-types. This complex
766 space has a “topology of the possible” (133), and the path of evolutionary trajectories

767 across this complex topology identifies the evolutionary topology of antibiotic resistance.
768 This topology, which lacks metrics, is hard to describe accurately; however, metaphoric
769 (mental) representations can help illustrate the possible paths of antibiotic resistance.

770 As represented in Figure 3, any evolutionary individual has a (clonal) descent; following
771 replication, any biological individual is an individual-in-time, an individual perpetuated
772 over time and transformed over time. This series of copies of the individual over time can
773 be represented by a cylinder, a tube that progresses in time. There are internal changes in
774 the clonal lineage (such as mutations) that provide changes, so that the trajectory of
775 changes occurs inside the tube (the space of variation), which might occur in synchrony
776 and sympatry with many other lineages. Different neighbor cylinders might exchange
777 characters by horizontal transfer (e.g., genes), which are now introduced into other
778 cylinders and influence the vertical descent inside these tubes. A set of tubes exchanging
779 adaptive characters should tend towards ecological convergence; for instance, the flow of
780 ARGs into different bacterial clones or even species tends to ensure coexistence of the
781 organisms in the same antibiotic-polluted environment, increasing interactive relations.
782 This process can occur in a single individual (e.g., in the gut microbiome) (135) or in a
783 higher hierarchical niche (e.g., wastewater plants) (136) and can be represented as a new
784 tube (meaning possible co-evolutionary trajectories) composed of related tubes, a
785 topological space that might be broken in other environments (Fig *XX*b).

786 As illustrated in figure XX, antibiotic resistance trajectories are multidimensional
787 trajectories that encompass a variety of evolutionary individuals at various levels of the
788 biological organization. This is in fact a processual ontology (137, 138) of antibiotic
789 resistance. The structure of this review is based on considering the evolutionary
790 trajectories of the various ontological hierarchies involved in antibiotic resistance.

Evolutionary Trajectories Interactions. The flow of evolutionary individuals occurs in a complex fitness landscape (see later) determined only in part by antibiotic exposure. A realistic description of evolutionary trajectories of antibiotic resistance should include a complex transhierarchical network of trajectories encompassing entities at various levels, from proteins to populations and communities (61, 139, 140). The evolution of antibiotic resistance should be necessarily compatible at any level of the hierarchy with other evolutions, other trajectories in search for numerous other types of adaptive advantages unrelated to antibiotic resistance. These adaptive needs can eventually conflict with the evolution of antibiotic resistance, and their paths might eventually converge during part of the journey (acquisition of traits that are advantageous for the adaptive needs of both organisms). For instance, traits favoring *E. coli* gut colonization, given the production of microcins (small antimicrobial peptides) are frequent in multiresistant clones, such as O25B-ST131. Antibiotics might eventually select not only antibiotic-resistant also but successful colonizer strains and vice versa (141), thereby decisively influencing antibiotic resistance. Adaptive trends unrelated to antibiotic resistance are extremely important in shaping resistance trajectories. In the phylogenetic diversification of a bacterial species such as *E. coli*, which is driven by its exposure to different environments (142), a number of groups have evolved (speciation-clonalization) in a way that has facilitated the acquisition of antibiotic resistance (143). Interestingly, *E. coli* phylogroups with smallest genomes (probably with a reduced intrinsic resistome) have the highest rates of gene repertoire diversification and fewer but diverse mobile genetic elements (144).

An adaptive gain, modification, or loss of metabolic pathways all influence antibiotic susceptibility (as a bactericidal effect) and resistance (145, 146). The evolutionary mutational paths toward antibiotic resistance are constrained by the type of nutritional

substrates available; on the other hand, antibiotic resistance traits might modify the bacterial metabolism; for instance, by a shift from a respiratory to a fermentative metabolism of glucose or through the use of alternative respiratory chains upon efflux pump overexpression (147–149). The bacterial metabolism is also determined by the coexistence with other species in small habitats (150). Bridging the gap between the cellular metabolism and the community metabolism of microbial communities embedded in a common "chemosphere" (141, 151) and its influence on antibiotic resistance mutational paths (or horizontal gene transfer) is an interesting line of research (147, 152) that might help detect their Achilles heel to specifically inhibit resistant organisms.

However, the evolutionary trajectories dominated by resistance (to antibiotics, biocides, metals) might have certain advantages over other trajectories, given that the selective effect is stronger. Observations in other fields have suggested that, in case of conflict, the evolutionary side that can survive and grow at the expense of others (antagonism) is able to adjust the variable in its preferred direction (153). In summary, the evolutionary trajectories of antibiotic resistance are not only dependent on antibiotic exposure (selection) but also the absolute fitness of the evolutionary units (organisms, mobile elements, communities) involved in the process (154). Interactions (such as competition) between trajectories might occur between successive alleles on the *same* trajectory; variants with a high initial fitness might have less fitness later and might be outcompeted by other variants (155).

The Question of Causality in Evolutionary Trajectories. The classic meaning of "evolution" implies that a biological (or genetic) entity undergoes progressive and cumulative changes to become, above a critical threshold, a different entity (ontology). The term "trajectory" includes the description of the successive evolutionary steps that determine the path a biological entity takes when moving from one significant ontology

841 to another; however, trajectories are more than just predictable chains of events. The
842 standard notion of an evolutionary trajectory requires that these changes have an order,
843 logic, and regular path determined by a necessity (by fitness?). As we will see in this
844 review, anisotropic evolutionary trajectories can be traced not only by necessity but also
845 by the interplay of determinism and randomness.

846 Biologists (and not only biologists!) tend to believe that changes are accompanied by a
847 force causing them. However, it has been proposed that there is a spontaneous tendency
848 for evolutionary individuals to differentiate, resulting in diversity and complexity arising
849 from the simple accumulation of random accidents. This "zero-force evolutionary law"
850 states that in any evolutionary system in which there is variation and heredity there is, in
851 the absence of constraints, a random tendency for diversity and complexity to increase
852 (156). Following the second law of thermodynamics, randomness in the molecular
853 evolution of bacterial sequences increases over time (157), and bacterial diversification
854 has generally increased continuously over the past billion years (158).

855 Randomness (chance) can be treated probabilistically (probability to determine);
856 however, there are frequently multiple evolutionary trajectories linking two points in the
857 evolutionary process, and the frequency of each of these trajectories depends on the local
858 factors influencing the fitness landscapes. In antibiotic resistance, the distribution of the
859 fitness effects of random mutations is highly variable among antibiotics, as has been
860 detected by high-throughput fitness measurements for genome-wide *E. coli* gene deletion
861 strains (159).

862 **Are there Random Trajectories? Stochastic "Drift" Evolution.** Antibiotic
863 resistance evolves through processes that involve determinism, stochasticity, and random
864 drift. "Drift" evolution implies that a number of variant phenotypes (in this case,
865 resistance phenotypes) in a population have emerged and spread by reasons completely

unrelated to the microorganism's adaptive needs when exposed to antimicrobial agents or to other adaptive needs. Experimental evolution studies have suggested that antibiotic resistant variants can evolve even in the absence of antibiotics, driven by the genetic adaptation of bacteria to various growth conditions in natural environments and hosts (152). These variants can be hooked by antibiotic selection and enriched by drift in small populations.

The most characteristic case of drift is random sampling. Take for example a population of identical bacterial cells with a tiny proportion of random resistant variants. Under antibiotic exposure, this resistant minority will be selected (antibiotics *determine* the disclosure of resistance). However, there is another way by which resistant minorities prevail. If the original population spreads into a large space (dispersal) or the population is broken because the cells colonize separate areas (such as the colonization of different hosts and the contamination of water and soil environments by sewage), the "resistant variant" cells might become isolated from the ancestor population and will produce a local resistant progeny in the absence of antibiotic selective pressure; a resistant population. In contrast, that we can also consider the opposite possibility: a homogeneously resistant population with a minority of "revertant" susceptible cells, which can give rise to susceptible populations. Drift can also remove resistant variants arising in susceptible populations (160). The noise created by drift might limit to a certain extent the refining activity of natural selection on particular phenotypes (drift-barrier hypothesis) (161).

In the first edition of "On the Origin of Species", Charles Darwin indicated the possibility of fluctuations in the frequency of variations with no adaptive significance, at least at the moment of their emergence (162). Paradoxically, such observation was the ground stone of the concept of non-Darwinian evolution (163). Sewall Wright was the first to attach

this significance to random drift and small, newly isolated populations through his shifting balance theory of speciation: the Wrightian modality of evolution, presented by Sewall Wright in 1932 during the Sixth Congress of Genetics in his seminal lecture on 'The roles of mutation, inbreeding, crossbreeding and selection in evolution'. Ernst Mayr subsequently created convincing models to show that the decline in genetic variation and small population sizes following a local invasion across a bottleneck were critically important for the development of new species (generally taxons). Drift, stochastic introgression and hybridization events produce "hopeful monsters", overcoming the need for gradual changes in evolutionary trajectories (164–167), eventually giving rise to high-risk resistant bacterial clones.

Dispersal and spatial structuration as sources of drift. Dispersal provides adaptive chances for minorities. Random drift is frequently presented as a sampling effect, such that the sampling of a population at different locations might yield differing results in the frequency of particular variants. If the frequency is the same, then the sampling number in each location is likely above the effective size of this population (the number of cells in a sample that faithfully capture the genetic diversity of the whole population). In other words, reduced populations should yield increased genetic drift. Large bacterial populations mostly evolve deterministically, whereas small populations follow more stochastic evolutionary paths (168). Drift is a powerful process in the formation of species (169, 170), which is also true for the clonalization processes inside bacterial species.

Bacterial dispersal distributes small populations over space, eventually leading to spatially structured populations colonizing different environmental patches. These "fragmented populations have evolutionary possibilities that are lacking in the original dense population. For instance, a genetic variation allowing access to an antibiotic-

916 resistance phenotype might have a significant biological cost when competing with the
917 wild progenitor population. The cells containing this will therefore be prone to extinction
918 in the absence of antibiotic selection. Laboratory microbiologists knows well that
919 particular mutants can be detected by spreading dilutions of the sample on culture plates
920 (creating spatial isolation), in contrast to broth tubes where the fittest mutant eliminates
921 the others. Given that competition is not an issue in spatial isolation, the resistant
922 population can grow and even achieve better fitness by compensatory evolution, retaining
923 the resistant phenotype. Drift is a diversifying process that takes advantage of small
924 populations as much as it is a mutation, an event that takes advantage of dense
925 populations. In both cases, new "selectable variants" are offered to antibiotic selective
926 forces.

927 When is drift evolution of antibiotic resistance expected to occur in practice? The main
928 conditions are a reduction in population size by spatial-temporal fragmentation and
929 opportunities for growth of the reduced groups in favorable patches, forming
930 metapopulations. Antibiotic exposure will then select for local drift-revealed resistant
931 populations. Drift evolution can therefore be interpreted as a form of metapopulation
932 dynamics. Metapopulations do not necessarily result from single cells, given that bacterial
933 dispersal might resemble Lévy dust, with a range of fractal patterns, from dispersed to
934 clustered ones (171–173).

935 **Fragmentation of infective populations, drift, and resistance.** Reductions in
936 population sizes occur due to a number of factors in infective-transmissible processes.
937 First, in *host-to-host transmission* processes, a small *propagulum* of cells serves to initiate
938 colonization or infection in each new host; thus, a spatially-structured fragmentation of
939 the original population should occur. Second, further fragmentations occur *within the*
940 *individual host*, where bacterial invasion is necessarily linked with the dilution of the

941 offending organisms in different compartments, tissues, and cells, and inflammatory
942 processes frequently lead to sequestration of bacteria in particular locations. Drift-
943 generated resistant variants might therefore eventually multiply locally. An increase in
944 the number of colonizable subhabitats is expected, especially in chronic infections and
945 when foreign bodies are present, the increase the number of colonizable sub-habitats is
946 an expected issue. Biofilms, which are frequent in chronic infective processes, provide
947 spatial structuration of bacteria, facilitating the drift evolution of resistance (174) and
948 diversification at large (175). The biofilm-planktonic interphase can trigger divergent
949 evolutionary pathways (e.g., those involving efflux pumps and antibiotic target genes)
950 (176, 177). Third, the release of human and animal sewage and other wastewater into the
951 environment produces dilution and population fragmentation. The attachment of bacterial
952 cells to soil particles (178) and organic remains increases the frequency of independent
953 mini-patches. Fourth, the use of antimicrobial agents and their release into the
954 environment can reduce the size of bacterial populations and promote the evolution of
955 drift-promoted resistance to different antibiotics, even in the absence of selection; this is
956 certainly an scarcely-treated topic. Fifth, fragmentation can occur due to asymmetric
957 (specific) mechanical forces, affecting bacterial cell adhesion to particular surfaces (179).

958 **Drift, draft, and trajectories.** In principle, drift is a chance and contingent effect,
959 and its contribution to evolutionary trajectories is nondirectional, following a type of
960 random "Brownian motion" in the evolutionary space, highly susceptible to extinction
961 events (180). As stated before, the contribution of drift to antibiotic resistance is akin to
962 that of mutational events, offering random genetic variants to the hook of selection. Drift
963 might therefore complement directional evolution mediated by successive adaptive
964 benefits, providing random solutions in broken adaptive trajectories (181). Fitness plains
965 and valleys (and not the peaks) are the territory of drift (182), where low density,

966 neutrality. and near-neutrality dominate, providing the substrate for adaptive and hidden,
967 preadaptive evolution (178). (Figure 4).

968 Why do rare neutral or preadaptive random variations not disappear in bacterial
969 populations? As Fisher and Haldane postulated in the 1920s in their theory of mutation-
970 selection balance, the answer lies in the immense number of bacterial cells and the
971 heterogeneity of the fitness landscapes in which these bacteria disseminate. Neutral
972 variation can "persist long enough" to allow the bacteria to reach an favorable
973 environment (a peak in the fitness landscape) by chance (albeit with a small probability),
974 an environment in which the bacterial organism carrying the variant trait has an advantage
975 over its competitors (e.g., a selective antibiotic concentration, resulting in an increase in
976 number and fixation of the trait). This "persistence of neutrality" appears to require large
977 populations, providing sufficient numbers to deal with the low probability of selection-
978 fixation. High numbers might favor a considerable multiplicity of small, isolated
979 populations across variable fitness landscapes, where drift dominates. Weak mutations
980 have a chance of being fixed only in small population sizes, given there is no competition
981 with more efficacious changes. However, even if a large population is not dispersed in
982 small populations, neutral variation can be maintained because it is randomly linked to
983 selectable traits and frequently "hitchhikes" with those loci subjected to directional
984 selection. That means that the adaptive variation in a selectable locus can therefore induce
985 stochastic dynamics (resembling genetic drift) at a closely linked neutral locus. This
986 hitchhiking, termed "genetic draft", has been proposed as a stochastic force analogous to
987 genetic drift (169, 183).

988 The randomness of early stages in many evolutionary trajectories leading to antibiotic
989 resistance is a consequence of drift, but once the adaptive trajectory starts, with low
990 increases in fitness, directionality (selection) eventually tends to be imposed. This feature

991 indicates that contingency is a major driver of stochasticity toward determinism in the
992 evolution of antibiotic resistance (112, 184). Genes near to those that are selected are
993 preferentially hitchhiked (linked selection, draft) and increase in number, thereby
994 increasing their chances of providing material for novel adaptations, related or not to that
995 of the "driver" gene. The strength of the directional selection at these early stages depends
996 (according to the classic Lande equation) on the product of additive genetic variance and
997 the selection intensity for the evolving trait (185).

998 **Genealogical, Across-Network, and Spinning Trajectories.** Adaptive trajectories
999 follow the fundamental tenet of evolution, the Darwinian principle of "descent with
1000 modification" (186), indicating the permanence along replications (time) of a common
1001 genetic patrimony and the fact that deviations from this heritage occur, either by
1002 modification in the individual or by acquisition of foreign traits.

1003 The study of phylogenies is therefore essential in classifying evolving individuals by
1004 similarities and tracing the process (trajectory) of their relationship with common
1005 ancestors. A limitation in the current phylogenetic (genealogical) analysis is the bias
1006 imposed in databases by the predominance of organisms of particular interest (such as
1007 those with antibiotic resistance) and the almost total absence of "real last-common
1008 evolutionary ancestors" (143). The phylogenetic approach, which provides trajectories
1009 within tree-like patterns, might indicate the presence of an evolutionary trajectory within
1010 a single progeny (genealogy) but should be considered an inspirational hypothesis but
1011 one that needs confirmation with actual data, a task that could be facilitated by automated
1012 phylogenetic tools (187).

1013 Phylogenies can be analyzed more accurately by superimposing them with other
1014 analytical methods, such as those that estimate the frequency of recombinatorial links
1015 between apparently separate (even distant) lineages (188) . Phylogenies reflect dynamic

processes, dependent not only on vertical descent but also on horizontal genetic interactions. This type of phylodynamics considers temporal changes in phylogeny under the influence of changing ecological contexts, which could have modified the original coalescent association between evolutionary units (such as genes and species). This view applies the coalescent theory analysis, in which both the "ancestor past" and the present are considered in order to trace the population shifts (189–191). Applications of this approach to the evolution of antibiotic resistance (in particular to resistance genes in variant clones) have already be developed (192, 193).

The basic problem with this approach is that lineage-only based phylogenies of bacterial organisms are likely corrupted in nature by the high frequency of introgressive events, leading to the stable integration of genetic material from one bacterial species into another. Horizontal gene transfer is essential for "building the web of life" by associating different genealogical lineages (57), which is true for every evolutionary individual along the hierarchy, from genes to communities. Transmission events occur at all levels, and recombination occurs at all biological levels (194). The representation of these intergenealogical branches does not produce a more tree-like pattern but rather a reticulate network pattern, which likely reflects the space of evolutionary trajectories in a more integrative manner (195, 196). (Figure 6).

However, network thinking is becoming increasingly more influential in evolutionary studies. Network-based approaches, such as sequence similarity networks, gene networks, genome networks (including core genome, accessory genome, and regulatory genome networks), families, genus communities networks, and genome bipartite graphs are frequently employed in evolutionary studies (197).

Are "tree-phylogenetic" and "network-reticular" trajectories mutually exclusive? In many cases, it is still possible to make robust phylogenetic inferences even in light of substantial

horizontal gene transfer (198, 199). Horizontal linkages can be hypothesized between vertical phylogenies, creating a superphylogeny. The linkages can be thought of as resembling distinct wool fibers, combed together with other strands, which are in contact with each other, create a single rolag (roll) of wool. Spinning produces the interwoven fusion of strands into a single evolutionary material composed of vertical and horizontal interactions, giving rise to a cord or spinning trajectories (200). (Figure 4). In summary, the complexity of biological systems, with multiderived causality and feedback in unpredictable contexts, makes it difficult to identify linear causations. Research should be oriented toward webs and networks of nonlinear causality (201).

Diversifying and Unifying Evolution in Antibiotic Resistance. Evolution progresses over time (the goal of evolution is the conquest of time); however, the dimensions in which the evolutionary process takes place might at first sight appear contradictory. Evolution, if not replication alone (156), leads to progressive diversification (diversifying evolution), i.e., producing numerous variants from a single structure many variants are produced, an "*ex unibus plurum"* disruptive dynamics. Resistance genes, transposons, plasmids, resistant clones, species, and communities are subjected to constant diversification, while these variants (or at least the variants that have survived) simultaneously tend to aggregate to form complex configurations with greater evolutionary possibilities (resulting in a unifying evolution), i.e., a single suprastructure emerges from numerous diverse structures, an "*ex pluribus unum"* integrative dynamics (180, 202). This is a biphasic universal game of rapid expansion-inflation and slow (but creative) contractions, resembling other evolutionary patterns in physics (203); in fact, this system has been presented as entropic and antientropic dynamics.

Diversifying or disruptive evolution is related to evolvability, given the diversity of configurations is the material required by evolution to find novel adaptive solutions.

Diversification, an analytical process, is based on variation and consequently provides enhanced possibilities for exploration of novel solutions when faced with changing or unexpected environments, increasing dispersal (migration) and access to new resources and taking advantage of disruptive drift. The fuel for bacterial diversification is replication, an *r*-strategy favoring reproduction in the spatial dimension (204) (180).

In terms of antibiotic resistance, the mechanisms of diversifying evolution (in addition to classical mutations in targets, transporters, and regulators) include mutational events in resistance genes providing spectrum-enlarged or more stable antibiotic-inactivating functions, mutations that increase evolvability (hypermutation), and those derived from increases in bacterial population size due to antibiotic-selective effects, including selection by low antibiotic concentrations. Selection-associated replication facilitates dispersal of resistance elements (such as clones, plasmids and genes), random drift effects, and interaction with heterogeneous environments, which eventually enables interactions with elements of other bacteria, all of which increase the resistance gene, plasmid, and clonal diversification. Exposure to unexpected environments, including exposure to host defenses, other antibiotics, biocides, heavy metals, and bacterial phages increases stress responses, which in turn likely primes resistance gene mutation, amplification, and recombination, altering modularization at large and fostering diversity (including that involved in antibiotic resistance). Clear examples of diversifying evolution occur in the natural history of particular families of beta-lactamases and in the continuous emergence of novel splitting sub-clones and sub-sub-clones as a result of the expansion of a particularly successful clone. The final image is of an increasingly loose network reflecting increased dissemination in space and reduced penetration in time for each variant.

Unifying or integrative evolution should improve robustness, i.e., a configuration's ability to tolerate changes that might become deleterious for survival. Unifying evolution ensures stability, long-term exploitation of resources despite alterations, and niche construction and might increase the selection of integrated "wholes". Unification is favored by a type of "nostalgia of the ancestor", the homesickness attraction of the benefits in the founder niche (205). Unification is a synthetic dimension, where evolution improves the quality and efficiency of the evolutionary constructions, obtaining all possible advantages from the exploited area; in this sense, it is a *K*-strategy, favoring reproduction in the temporal dimension. Both dimensions are pivotal in the evolutionary "density game" theory (206).

The mechanisms of unifying evolution in antibiotic resistance include antibiotic selective events as strong reducers of diversity, thereby ensuring the success of a limited number of genes, plasmids, and clones. Genetic diversity is also eventually reduced by mechanisms that reduce resistance mutations (such as DNA repair systems), focusing mutational events on segments (207) and stress-attenuating mechanisms (208), including gene silencing. Diversity is also hampered by mechanisms controlling the uptake or maintenance of foreign DNA, such as restriction-modification, resistance plasmid surface exclusion and incompatibility, restricted host-range of mobile genetic elements, and clustered regularly interspaced short, interspaced repeats (CRISPR). Unifying or integrative evolution is not only driven by a reduction in diversifying or disruptive evolution. Horizontal gene transfer (and eventually recombination) is facilitated among members of the same lineage (kin) unlike with distant ones (see below, XXX). Thus, related lineages that have been subjected to diversifying evolution, which might have independently collected certain genetic traits involved in antibiotic resistance, can subsequently share such genetic material, which is collected within a single clone or a

bunch collection of kin lineages. The final result is a dense network of shared traits, facilitating integration and robustness.

The building-up of complex genetic structures of resistance elements is favored by modularization, eventually facilitated by lateral and intrareplicon integron dynamics, (209). Evolutionary convergence of previously divergent lineages can be modeled, including arbitrary split systems in sequence evolution models (210). Disruptive and unifying evolution compete, producing various types of constraints, such as evolutionary processes (211). The r/K selection theory indicates that bacterial populations might reach a certain equilibrium (trade off) between disruptive and unifying evolution, ensuring a balance of reproduction (quantity) and carrying capacity with complex local specialization (quality). This equilibrium has been predicted to occur in antibiotic-resistant populations (212). These disruptive-integrative evolutionary dynamics imply the possibility of breaking robustness (leading to a novel round of diversification). In complex network systems, there is the possibility of asymmetrical dynamics in one part of the complex, giving rise to system clashes (213).

STEPS ALONG ANTIBIOTIC RESISTANCE PATHWAYS: SOURCES AND FREQUENCIES OF VARIATION

Phenotypic Variation: Bet-Hedging Adaptive Strategies

The isogenic offspring of a bacterial cell offer a wealth of non-inheritable variability, phenotypic diversity. Variability is the source of evolution (214). In bacterial populations, the continuous emergence of minorities of phenotypic variants produces significant phenotypic heterogeneity (plasticity), which, due to the subdivision of risk-spreading, helps increase the lineage's chances of survival when confronted with unpredictable environmental fluctuations (215). In most cases, the origin of the heterogeneity appears

to be derived from “noisy gene expression”, random epigenetic interactions, gene amplification (see later), and, with less stochasticity, reversible stochastic switching of gene expression (bistability) (216, 217). Heterogeneity in gene expression increases genetic variability, particularly in poor growth conditions (218). Transient gene silencing, which frequently involves frameshift mutation, is not infrequent in resistance genes (219). Such “noisy gene expression” might itself be conceived as a selectable trait tunable by evolution, given that its excesses are regulated by dosage compensation in gene networks (220). There should be a certain “cost of high phenotypic variation” dampens dampening the strength of selection toward phenotypic heterogeneity and promoting directional selection of certain trajectories (221). However, a high rate of phenotypic heterogeneity is a safe “emergence strategy” for bacterial survival, but the advantageous phenotypic variants do not necessarily guide the directionality of the genetic adaptive trajectory (222). Such strategy, which has been presented as “bet hedging”, where certain phenotypes are selected in differing conditions and times, even though in most other cases the phenotypes can reduce the variant’s fitness (223–225). The bet-hedging strategy occurs in antibiotic heteroresistance, when a minority “resistant” subpopulation is present within a main population of susceptible cells (226). This strategy is not conceptually different from reductions in susceptibility by decreased growth rates as a method for reducing antibiotic stress (227). This reduced growth is mediated by genome inversions and reversible stochastic but self-organized inversion switches, which also affect antibiotic resistance, ensuring adequate time to develop compensatory mutations following the emergence of a resistant trait (228–230) and the stochastic emergence of persister cells with high resistance to the antibiotic killing effect (231, 232). However, environmental variation in time or space is not a necessary condition for the evolution of phenotypic heterogeneity (221).

The emergence of metabolic heterogeneity in isogenic bacterial cells with various growth rates has been observed (233), with possible consequences for antibiotic susceptibility. The regulation of adaptive phenotypic resistance under stress has extensive interpopulation and intrapopulation heterogeneity; however, the stress-adaptive genes with lower expression variability appear to have greater impact on adaptation (234). To what extent this phenotypic variation influences the evolution of heritable antibiotic resistance is an interesting topic (235).

Particular phenotypes (reflecting physiological states) might indeed facilitate the survival and selection of a fraction of the bacterial population under antibiotic exposure. For instance, phenotypic fluctuation in outer membrane proteins, membrane charges, molecules involved in protein synthesis, mistranslation, and expression of pumps might reduce antibiotic action and produce phenotypic resistance. In many of these cases, the evolution toward inheritable resistance (by mutation or horizontal gene transfer) might be facilitated, just by maintaining a critical population size (216). The persister populations in the gut, which can periodically recolonize, might serve as a reservoir of antibiotic-resistance plasmids (236).

Functional or genetic variations of the global regulators (as ArcA or RpoS) probably play an important role in global one-step adaptation (237). Micronutrition and other environmental effects might affect the evolution of antibiotic resistance. For instance, overexpression of iron storage proteins, inhibition of iron transport, and anaerobic conditions that alter oxidative damage-induced mutagenesis were found to suppress the evolution of fluoroquinolone resistance (238). In certain cases, “bet-hedging” can also act as a nonstrategy, governed only by fortuitous (not inheritable) errors in cell replication, resulting in transient periods of nonreplication and/or slowed metabolism, as likely occurs

in certain "persister phenotypes" (239). Such "cellular noise" is likely amplified by epigenetic inheritance, stochastic transmission of proteins, RNAs, and other biomolecules from parent to offspring cells (240).

We previously discussed errors in translation as a source of "phenotypic mutations". Amino acid misincorporation during translation produces mutated proteins that might produce novel functions, including antibiotic resistance. Erroneous protein synthesis might affect the protein's specific activity, such as misfolding and stability, with possible phenotypic consequences. The frequency of noncognate amino-acid incorporation is as high, in the range 10^{-4} as 10^{-5} One-fifth of proteins produced in a given cell contain at least one wrong amino acid (241); however, considering the proteins' short lifetime, these changes can have phenotypic consequences over a short period (242), which can be sufficient for expressing an antibiotic-tolerant phenotype, particularly in conditions of slow growth (243). Counterintuitively, translation mistakes might have fitness-enhancing consequences, positively influencing antibiotic resistance (for instance, exacerbating the effects of deleterious mutations and facilitating their purging and/or stabilizing changes and increasing the trait's robustness) (65, 244).

In summary, phenotypic noise is a potentially important factor in evolution (12). Phenotypic plasticity and fluctuation accelerate evolutionary rates in multipeaked landscapes (Baldwin effect) (245). Later in this review, we will discuss how stress produced by antimicrobial agents (or other causes of stress) can enhance phenotypic variation and consequently the evolution of antibiotic resistance.

Mutations Leading to Antibiotic Resistance

Mutation rates. Mutation essentially depends on the error rate of replication set by the accuracy of DNA polymerases and various DNA repair systems. In most DNA-based microbes, the mutation *rate* ranges from 10^{-10} to 10^{-9}/cell/generation, depending

on the specific substitution, gene, and organism and considering selectively favorable, unfavorable, or neutral mutations. This rate is approximately 10 times lower than the typical *frequency* of mutation (e.g., 10^{-8} for *E. coli*), which measures all mutants present in a given population as those surviving a given antibiotic concentration (246). The lower limits for mutation rates might be set by the costs of maintaining high-accuracy DNA polymerases and repair systems. It is also possible that the evolution of mutation rates results from the interplay between natural selection (primarily operating to improve replication fidelity) and the limits of what is possible, imposed by random genetic drift (247).

Mutation per species, gene, and day in a single host. Simple calculations offer an intuitive image of the mutation frequency in natural populations. The *E. coli* genome has approximately 5000 genes, and the mutation rate for wild-type *E. coli* is 1×10^{-3} per genome (cell) per generation (248, 249), which, divided by the number of genes (0.001/5000) yields $2x10^{-7}$ per gene and (cell) generation. If there are 10^9 cells/ml in the colon in a volume of 1000 ml, there would be 10^{12} *E. coli* cells in a single host, implying that there are 200,000 mutations per gene per day (1 generation) for *E. coli* in a given host. Given that particular *E. coli* clones are frequently stable colonizers of the gut, thousands of generations will amplify the total number of possible mutations. Similar calculations regarding the rate of evolutionary change have been recently discussed in relation to the gut microbiome (250). Considering this enormous mutational load, most genes display remarkable stability over time, which is most likely due to purifying selection, i.e., the alleles produced by mutation are selectively removed if they are deleterious and do not expand unless they are advantageous. Gene stability is also due to the effects of genetic drift, by which novel alleles are randomly lost due to the frequent bottlenecks that bacterial populations encounter. The result is stabilizing selection

through the purging and loss of not only deleterious variants that arise in the population but also of the linked neutral sites. The removal of a particular allele might also reduce the diversity of other linked neutral alleles by linked selection or background selection (251). A number of examples have shown that these calculations are roughly correct under *in vivo* conditions (252). Take for example the potential mutational wealth of a single individual with a chronic infection (such as cystic fibrosis) in whom 200,000 generations of a single organism (*P. aeruginosa* or *Staphylococcus aureus*) can be traced over a number of years (253). However, these figures and frequencies are insignificant when extrapolating to the total number of prokaryotic cells on Earth (estimated at $5x10^{30}$).

The rate of mutation per gene is not linear across the chromosome; there are genes and regional mutational hotspots such as slippage contingency sequences (112, 207). Localized hypermutable sequences are frequently involved in multigene phase variations that affect the outer membrane, restriction systems, and antigenic variation resulting from recombination events. These privileged variations have been shown to compensate for the limitations of host-to-host transmission bottlenecks (254). Mutation densities are greatest in regions predicted to have high superhelicity (255). On the other hand, a mutation that potentially provides resistance does not necessarily result in a "phenotypically effective mutation", particularly in rapidly growing cells. Polyploidy derived from multiple replication forks might produce a phenotypic delay of a recessive, antibiotic-resistance mutation that remains undetectable during the next 3–4 generations (256, 257). This is particularly the case for plasmid recessive mutations, because plasmids can be regarded as stable polyploid DNA molecules (258). In any case, our current ability to detect rare mutations in the global sequence space that potentially provide antibiotic resistance is probably low. However, methods of directed evolution with random genomic

mutations that allow for an up to one-million-fold increase in the mutation rate have been recently proposed (29).

Hypermutation in the evolution of antibiotic resistance. Increasing mutation rates can be expected to offer a wealth of novel mutations that eventually can produce selectable phenotypes, such as antibiotic resistance. If the environment changes rapidly, includes stressful conditions and bottlenecks, and is highly compartmentalized, variants with increased mutation rates (mutators) tend to be selected, given that they have an increased probability of forming beneficial mutations. Approximately 1% of *E. coli* strains have at least 100 times the modal mutation frequency of 10^{-8} (strong mutators). A very high proportion of strains (11–38% in various series) had frequencies exceeding 4, in some cases 40 times, this modal value (weak mutators) (259, 260). Hypermutation is frequently due to the impairment of the mismatch repair system and, more specifically, that involve alterations in not only the *mutS* gene but also *mutL* and *mutH*. Weak mutators might also result from variations in the DNA translocase protein Mfd, interacting with RpoB and UvrA interactions, leading to an accelerating evolution of antibiotic resistance (261).

A mutator allele and its potential beneficial mutations arising from hypermutability are physically and genetically associated in the same chromosome. As a result, the mutator allele will hitchhike with increased frequency in the population together with the beneficial mutation. Mutators are fixed in competition with nonmutators when they reach a frequency greater than or equal to the product of their population size and mutation rate (262). In populations of sufficient size, advantageous mutations tend to appear in normomutators, and the selective process will therefore enrich low-mutating organisms. Eventually, the adaptive success of normomutators might prevent further fixation of strong mutators. Hypothetically, in very large bacterial populations, the likelihood of

normomutators providing a substantial number of mutants might be sufficiently high, such that any additional increase in the mutation rate might be considered as relatively irrelevant. However, we should consider the frequent spatial compartmentalization of bacterial populations (including biofilms), where the *total* population size frequently has little relevance for the *local* adaptive needs of relatively isolated smaller subpopulations, in which the emergence of a mutant might be critical. It has been suggested that the emergence of antibiotic resistance might accelerate in connected microenvironments (263, 264). Antibiotic gradients create a compartmentalization of differing selective antibiotic concentrations (265). The bacterial population thus exposed to a particular concentration (in a particular space) can be relatively low. Antibiotics (or innate immunity during infection) by themselves reduce bacterial populations, so that high mutation rates in the residual small population of survivors can have adaptive importance. In general, mutators are not more "creative" than normomutable strains in the search for beneficial mutations (or trajectories); they just reach the advantageous outcome sooner. The mutation supply rate affects the speed (tempo) but not the pattern (mode) of evolution (266). However, not all mutators are equally likely to produce a given mutation. This bias emerges from the molecular mode of action of the mutation correction system that is disrupted in each mutator genotype. For instance, inactivation of the mismatch repair system in *E. coli* leads to a specific ~100-fold increase in G:C → A:T and A:T → G:C mutations (267), which has profound implications for competitive ability of mutators and determines their evolutionary success (268). Experimental evolution research has demonstrated the possibility that the emergence of a mutator might occur under antibiotic exposure by the reversible insertion of a mobile element to inactivate *mutS*, resulting in several mutations independently able to increase resistance (at various levels) to a

challenging antibiotic in the population, thus providing an "efficient survey" of potentially successful evolutionary trajectories (269).

The same effect of "small populations" (bottlenecks) occurs when bacteria cross from host to host, in which increased mutation rates might be significant in the bacterial adaptation to novel habitats (254). The evolutionary advantage of small populations in complex fitness landscapes has been suggested by various authors (168, 270, 271). On the contrary, high population densities tend to reduce spontaneous mutation rates (density-associated mutation-rate plasticity) (272).

It has been shown, both in the case of mutation-based resistance (273) and in the evolution of resistant genes carried by mobile genetic elements (274), that antibiotic-resistant organisms frequently have increased mutation rates, which suggests the evolutionary consequences of hypermutation. Does the fact that organisms with mutator alleles can hitchhike with antibiotic resistant phenotypes indicate that the rise in antibiotic resistance might increase the evolvability of bacterial populations in general? Hypermutation should have an evolutionary cost, eventually leading (beyond an "error threshold", as proposed by Manfred Eigen) to an "error catastrophe" (275). The accumulation of adaptive mutations in a single resistance gene (and mutations in other parts of the genome), which is needed in order to deal with successive antibiotics of the same class or at higher dosages, might produce a type of error catastrophe, destroying the information of the encoded molecule(s) and/or increasing the amount of deleterious mutations unrelated to antibiotic resistance. Hypermutation provides a short-term fitness benefit for adaptation of antibiotic exposure but at the expense of an unbearable fitness cost (276). Thus, hypermutable variants might not persist, a feature that has been experimentally demonstrated (208).

The methods by which bacteria modify mutator phenotypes are certainly of interest (277). It has been shown that a number of hypermutable organisms evolve to phenotypes of normal mutation rates, eventually by reacquisition of the functionality of damaged mismatch repair systems or by the coincidental overexpression of mechanisms that reduce the endogenous mechanisms of mutation (188). Due to these effects, populations with lower fitness but more robustness to mutational effects might displace highly replicative hypermutable populations, which has been described as "the survival of the flattest" (278, 279). Experimental evolution shows that although populations with higher mutation rates increase genetic variation, the adaptive benefits of such diversity in novel environments might be lower than those derived from modestly increased mutation rates (280). These modest increases in mutation rate are more frequently found in clinical bacterial isolates than in higher ones (259).

Mutational events by insertion. Mobilization of insertion sequences (IS) in particular and transposable elements in general cause genomic variability in bacteria (281, 282); however, their influence on mutation rates and adaptive evolution is small compared with mismatch repair mutator alleles. There is competition between mismatch hypermutation and IS propagation by hitchhiking (283). Transposable elements are enriched by inserting extra copies in the host genomes, which might cause a certain conflict. Genomes have therefore evolved suppressors that limit transposon spread (130). The effect of IS on resistance gene mutational events is discussed later in this review.

Polyploidy and Gene Amplification: from Adaptation to Neofunctionalization

As discussed in the previous section, increased copies of a particular gene (polyploidy) should increase the possibility of mutational modification in particular and evolvability

in general. During the exponential phase of a fast-growing organism, a large number of copies (eight or more) of numerous genes are available; however, polyploidy also occurs in the stationary phase (256). Bacterial stress (including antibiotic stress) might produce cell filamentation and polyploidy (see Section XXX).

Gene amplification (gene duplication in its simplest version) is likely relevant in the adaptation to antibiotic exposure because it generates extensive and reversible genetic variation on which adaptive evolution can act. The steady-state frequencies of gene duplication are extremely high, typically ranging between 10^{-5} and 10^{-2} per cell per gene (90). Amplification produces a gene-dosing effect, increasing the transcription of a resistance gene. For instance, sulfonamide, trimethoprim, and beta-lactam resistance (including resistance to beta-lactam plus beta-lactamase inhibitors) occurs due to increased gene dosage through amplification of antibiotic hydrolytic enzymes, target enzymes, or efflux pumps (284, 285). Amplification of the *vanM* gene cluster (acting in a similar fashion to *vanA*) in *Enterococcus* confers glycopeptide resistance (286).

The genes that are present in high copy number plasmids are also "amplified". These cells are now selectable in low antibiotic concentrations, increasing in number, and therefore increase the probability of new adaptive mutations, eventually leading to higher levels of resistance (287). Once this occurs, low-level resistance by amplification alone is no longer efficiently selected. Sequence amplification provides rapid adaptation to antibiotics but is evolutionarily costly (288), and gene amplification is inherently unstable (289). Fitness costs have been evaluated, and each additional kilobase pair of DNA reduces fitness by approximately 0.15% (290), resulting in amplification returning to the original single-gene status. Recent detailed studies on *E. coli* and *Salmonella typhimurium* have shown that gene duplications in a size range of 20–1246 kbp are associated with costs on the order of a 0.05–1.5x10^{-3} reduction in fitness per 1 kbp of

extra DNA (290, 291). No signal of this transient event will remain in the genome sequence, which is why this evolutionary mechanism remains underdetected (292). However, the high prevalence of antibiotic heteroresistance in pathogenic bacteria is most likely caused by gene amplification (293).

Gene amplification is also a source for the evolution of new functions (294). Once the adaptive requirement is over, the duplicated gene will most likely be lost or subjected to nonfunctionalization by the accumulation of mutations. Subfunctionalization is possible, in which both copies acquire neutral or quasi-neutral mutations; however, the two partially functional genes complement each other. Lastly, the acquisition of a novel function, neofunctionalization, occurs if one of the duplicated copies acquires a novel (selectable) function while retaining the old function in the other copy (90, 290, 295–298). In fact the "Ohno's dilemma" indicates that if a gene duplication is selected because of an increase in the original function of the original single gene then the copy is not free to be selected for any other novel function. The dilemma can be solved with the enrichment by selection of the total number of copies under continuous selection (299, 300) . Eventual amplification of a resistance gene might severely reduce the fitness of the strain, both in the presence and absence of the drug it counteracts. For instance, an excess of the Tn*10*-encoded tetracycline resistance protein, TetA, produces a partial collapse of the membrane potential in *E. coli*, eventually resulting in cell death (301).

Gene amplification also has consequences on bacterial chromosome rearrangements, given that recombination between duplicated sequences are expected to produce partial chromosomal duplications, with negative or positive consequences on fitness but eventually facilitating access to novel niches where new chromosomal arrangements can be fixed (302).

An interesting topic is the role of mobile genetic elements in evolution through polyploidy or gene amplification. Self-replicating mobile genetic elements control their own copy number in the host cell. Some of these elements, such as the ubiquitous small multicopy plasmids, usually present 10–20 copies per cell. Plasmids therefore represent a potential platform for the neo-functionalization of genes that could easily overcome Ohno's dilemma (303). A high number of plasmid-born gene copies would allow bacteria to explore new functions through mutation while conserving the functional backup of several copies of the gene (304). In multicopy, plasmids might result in a "growth with amplification" SOS-induced mutagenesis (305). In addition, a number of plasmids encode for error-prone polymerases (as DinB in the F9lac plasmid). In multicopy, these plasmids might increase the evolvability of both plasmid and chromosomal genes (292, 306, 307).

Horizontal Gene Transfer

The genes subjected to horizontal gene transfer. Horizontal gene transfer provides the theoretical possibility for each gene of the biosphere to enter into contact with the genome of any bacterial organism. There is an estimated 10^{10} to 10^{12} genes producing different structural and functional properties (90, 308). Considering that studies on the intrinsic resistome indicate that the percentage of resistance genes in any microorganism falls within 1–3% (31, 38), a conservative evaluation would indicate that there are 10^8 different genes in the world capable of conferring resistance to antibiotics, a number obviously beyond our analytical capability. Mutations in many genes could contribute a resistance phenotype for a particular antibiotic. For instance, it has been shown that 135 genes reduce susceptibility to tobramycin in *P. aeruginosa* (309) and therefore are putative resistance genes. Hypothetically, this enormous collection could form a microbial common good, providing outstanding collective plasticity to the microbiosphere. This potential commonality is based on the fact that even remote

possibilities might occur, sustained by the astronomically large number of bacteria estimated to exist in the world: 3^{29} cells (310).

Not all genes have the same possibility of being transferred. The number of genes of foreign origin (putatively acquired by lateral gene transfer) can be inferred for each group of bacterial organisms by considering the core genome, the ensemble of genes that are constantly harbored in all members of the group, typically a species. In contrast, the accessory genome reflects the ensemble of genes that have been acquired and retained to adapt subgroups (typically clones) to particular environments. The study of the historical phylogeny of bacterial pathogens, such as *Yersinia pestis*, has shown that the acquisition (and loss) of specific genes is the basis of bacterial speciation (311). Nevertheless, evolution toward antibiotic resistance is a recent event in evolutionary terms, and speciation is not an expected outcome of the acquisition of resistance.

In a strict sense, ARGs (not including wild or mutated genes providing physiological functions) belong preferentially to the acquired (accessory) class of genes (13, 312). In the genes carried in mobile genetic elements, the proportion of genes associated with antibiotic resistance is uncertain because databases provide a biased sample of the species; however, the proportion should be high in clinical isolates (313). Curiously, diverse broad-host-range plasmids in nature carry few accessory genes (314).

ARGs arriving at a particular microbial organism by horizontal gene transfer without providing any further adaptive advantage besides resistance might not be permanently integrated in the new host's genome, given that integration affects genome organization. Transferred genes are concentrated in only approximately 1% of the chromosomal regions (hotspots) (315), which is likely one of the key roles of extrachromosomal elements in integrating adaptive genes. Even if accepted, the genes might be unable to function as significant pieces of information, such as providing an antibiotic resistance

phenotype. Disparity in codon usage between the donor and recipient organisms can influence gene translation efficiency and might impose a fitness cost for the receptor.

Gene capture by transposable elements. There is a large spectrum of related transposable elements that are vehicles for ARGs. In addition to resistance genes, transposons might carry other adaptive elements that can help in the selection of antibiotic resistance. Notably, the Tn3 family of transposons can capture (or evolve) entire operons, with resistance to heavy metals (such as mercury), antibiotic resistance, breakdown of halogenated aromatics, or virulence (316). Heavy metals are the most abundant pollutants worldwide, and heavy metal pollution has a historical record that begins with early mining activities. The early acquisition of heavy metal resistance genes thousands of years ago, as a consequence of mining, might have helped the expansion of a specific subset of gene capture and mobilization elements that now form the task force in acquiring and disseminating antibiotic resistance, as an example of the relevance of contingency in shaping antibiotic resistance evolution.

Genomes in turmoil: gene acquisition, gene loss. Horizontal gene transfer and the integration of these genes in the host genome is a frequent process in nature, resulting in a constant and variable flux of genes in bacterial organisms. The effects of transposable units such as IS include massive expansion and loss of DNA fragments, producing gene inactivation and decay, genome rearrangements, and genome reduction (282). How is this turmoil tolerated? There should be a way of regulating the genome's optimal size. Frequent horizontal gene transfer leading to genetic innovation is probably compensated for by highly frequent gene loss, leading to genomic contraction. Eventually, gradual but significant gene loss is compensated for by episodes of rapid gene gain (317). This process is influenced by the fact that gene loss favors intergroup collective actions, such as cross-feeding, which requires contiguity, a condition for gene gain (318). It can be

argued that the acquisition of high pathogenicity and antibiotic resistance islands could be favored in variant clonal backgrounds having experienced genome reduction. Genome reductions generally occur in the accessory genome but can also occur in redundant genes and genes that are no longer needed when bacteria enter a new host/habitat. An interesting case is the loss of a copy of an rRNA operon in methicillin-resistant *S. aureus* in association with the acquisition of antibiotic resistance (319). However, these reductions might produce a significant stress and fitness cost, given that accessory or redundant genes are not fully dispensable and contribute to cellular physiological comfort, robustness, and adaptation to environmental fluctuations (320). Streamlining, however, is not necessarily the best evolutionary strategy (321). Occasionally, large chromosomal deletions might produce a growth advantage in the presence of an antibiotic, as in the case of *P. aeruginosa* and meropenem or ceftazidime resistance (322–324).

Transferable antibiotic resistance, recombination, and bacterial evolution. Does the anthropogenic release of antibiotics and the resulting spread of transferable antibiotic resistance act as a driver (accelerator) of microbial evolution? Under antibiotic exposure, genetic promiscuity is expected to increase. The transfer of resistance genes contributes to recombination between different replicons and, consequently, to their evolvability (136, 325). Mobile genetic elements carrying resistance genes frequently have site-specific recombination systems and IS, whose location either in plasmids or in chromosomes favors homologous recombination, thereby favoring different events of integration or excision and interplay among different elements *(17, 22–26 MicrobSpect)*. Recombination events are also expected to contribute to the long-term adaptations of resistant populations in changing environments (complex fitness landscapes) interacting with stochastic epigenetic variation (332). This collaboration of antibiotic adaptation and

environmental adaptation at large (the “evolving to survive” paradigm) should influence the natural history of resistant organisms.

The consequences of increasing recombination affect the evolution of resistance genes (for instance, favoring the capture of mutated sequences from a related gene, such as the *bla* and *qnr* genes) (167, 333).

Cells have a wide variety of protective mechanisms to limit dangerous recombination events originated by the acquisition of foreign DNA, even if such DNA might be helpful, as in the case of antibiotic resistance. Restriction modification (RM) systems and CRISPR, frequently located in "defense islands" in microbial genomes, are the main post-transfer sequence-directed immunity mechanisms protecting a given host cell from invasion by foreign DNA, either by conjugation transformation or transduction *(6, 27, 28)*. In particular, the Wadjet condensing-based mobile system is an effective barrier against foreign plasmids (337). Some RM systems specifically limit the acquisition of plasmids to some pathogens and can influence their clonal structure (338, 339) however, RM systemms are sometimes acquired as a selfish “mobile element” acting on genome evolution (340). The mismatch-repair system inhibits interspecies recombination, the inducible SOS system stimulates interspecies recombination, and natural selection determines the effective recombination frequencies (341, 342).

DRIVERS OF VARIATION AND SELECTION SHAPING TRAJECTORIES UNDER ANTIBIOTIC EXPOSURE

Stress and Antibiotics as Drivers of Genetic Variation

Stress-induced mutagenesis is a main driver of bacterial evolution (343). Antibiotics are not only selectors but also drivers of bacterial genetic variation. Antimicrobials produce

1533 stress reactions in the susceptible organisms, frequently at sub-inhibitory concentrations,
1534 during growth phases in which antibiotics are less active or during at least relatively short
1535 periods. Bacterial stress is likely the result of conflicting cellular signals: on one hand,
1536 positive signals “to grow”; on the other, signals indicating the “impossibility to grow”.
1537 Mutation rate can be increased by antimicrobials promoting the stress-induced SOS
1538 response, which modulates genetic instability (344). Subinhibitory concentrations of
1539 antibiotics then produce stress, and stress induces mutations. Various mechanisms can
1540 account for such a process. First, stress (including antibiotic stress at subinhibitory
1541 concentrations) frequently results in bacterial filamentation and cellular polyploidy,
1542 increasing the opportunities for mutational events (see section XXX). A number of
1543 antibiotics (mainly bactericidal) cause reactive oxygen species production, which induce
1544 the low-fidelity polymerase DinB (PolIV), increasing mutagenesis, as occurs in *E.*
1545 *coli*with beta-lactams (345). Small concentrations of beta-lactam antibiotics induce the
1546 RpoS regulon, reducing MutS availability, resulting in further mutagenesis and less
1547 mismatch repair (346). However, studies of evolvability under antibiotic stress at
1548 subinhibitory concentrations consider that these concentrations frequently produce slow
1549 growth and death in part of the population; increases in mutation rates could therefore be
1550 overestimated (347). Antimicrobial substances, including antibiotics and biocides, might
1551 act at subinhibitory concentrations as inducers of horizontal genetic transfer of resistance
1552 genes in bacterial populations (348) and among commensal organisms in the intestinal
1553 environment (349). There is a need for quantifying effective stress levels, occurring in a
1554 window of possible evolutionary rescue between no effect and extinction of stressed
1555 populations (350).

1556 **Antibiotics as Drivers of Populational Variation**

An important evolutionary consequence of antibiotic exposure deals is the changes in the population structure of microbial organisms. Evolutionary trajectories of antibiotic resistance depend on the selected resistant populations, because the final evolution depends on the interplay between antibiotic resistance and other adaptive traits of the strains, such as colonization of a particular host or epidemicity involving different hosts. Exposure to various "host ecotypes" produces evolutionary divergence in bacterial populations (351, 352). The acquisition of antibiotic resistance occurs in particular clones that are then selected and subsequently compete with and eventually replace others that remain susceptible.

Clonal replacement takes place through two main processes (65). The first is exogenous invasion, in which a resistant clone arrives at a particular host, colonizing the skin or mucosal surfaces, eventually increasing its absolute size by antibiotic selection and displacing other susceptible clones of the same or different species, thereby implying local clonal shifts. Exogenous invasion by a resistant clone does not necessarily require antibiotic selection if the clone is well-endowed with colonization factors. Invader strains generally succeed when their reproductive numbers exceed that of the background established strain; however, there are scenarios in which the less fit succeed in replacing the previous colonizer (353). The second process leading to clonal replacements is endogenous conversion. As in gene conversion, the term "conversion" in this context means that a successful biological entity *already established* in a particular environment acquires an adaptive trait present only in part of the analogous entities coexisting in the same setting, even in a transitory manner. In this case, antibiotic resistance enters into a well-adapted, high-density endogenous clone. Clonal shift is much less visible here; however, if the dominant clone increases its fitness because of antibiotic resistance, minority susceptible clones might be reduced in size. The dominant resistant clone will

eventually help restore a certain populational diversity by transferring adaptive traits to its kin, neighbor clones. In certain instances (typically in chronic infections in patients with cystic fibrosis), different clones can coexist within the same host (253, 354). The potential cooperation of these different clones in establishing a population-based phenotype of antibiotic resistance is a feature that has not been explored in detail.

Bacterial clones that succeed in acquiring both antibiotic resistance and a wide distribution are the high-risk clones, which will be analyzed in greater detail later in this review. These clones have been defined as highly specialized antibiotic resistant clones or clonal complexes (a clone with satellite clonal variants) with enhanced ability to colonize, spread, and persist in particular environments (particularly human-animal mucosal or skin surfaces). The clones are endowed with a diversity of natural or acquired adaptive traits, influencing epidemicity, pathogenic potential, and antibiotic resistance (352, 355). Paradigmatic examples include the penicillin-resistant clones in *S. pneumoniae*, in which resistance is concentrated in a few lineages, possibly because recombination is not constant throughout the overall pneumococcal population (356). Methicillin-resistant *S. aureus* (MRSA) probably originated through the transfer of SCC*mec* into a limited number of methicillin-sensitive *S. aureus* (MSSA) lineages (357); however, local invasions by MRSA cannot be ruled out (358). Similarly, a single clone named ST131 is primarily responsible for the global increase in multidrug resistance (MDR) among *E. coli* (359). These three examples illustrate how clonal expansion of a few clones could be a major contributor to the spread of antibiotic resistance.

Selection for Resistant Noninheritable Phenotypes

Evolution of inducibility of antibiotic resistance mechanisms. A number of antibiotic resistance mechanisms are inducible, i.e., they are expressed at a sufficient level only in the presence of an inducing agent, frequently the antibiotic substrate of the

resistance or a related molecule. Classic examples of inducible resistance are inducible penicillinase induction in Gram-positives bacteria such as *Staphylococcus* and *Bacillus*, (360) and macrolide resistance in Gram-positive bacteria (361) and *Bacteroidaceae* (362). In general, the inducibility of resistance genes at very low (subinhibitory) concentrations supports the hypothesis that antibiotics in nature act more as highly diluted deterrent "signals" between potentially competing populations than as real killing weapons, that is, they follow the ecological principle of "armament-ornament" duality (363–366).

The "inducer" effector molecule might be not the antibiotic itself but certain cell metabolites released as a consequence of antibiotic-cell interaction. For instance, the LysR-type transcriptional regulator AmpR activates the expression of chromosomal AmpC beta-lactamase in many Proteobacteria in response to changes in peptidoglycan (PG) metabolite levels that occur during exposure to beta-lactams (367). If AmpC is expressed in strains lacking AmpR (such as *Salmonella*), the biological cost is unsustainable (368). The presence of AmpR potentiates the evolution of beta-lactam resistance in *Pseudomonas*, an effect prevented by the combination of avibactam, an AmpC inhibitor (369). However, other types of resistance might emerge, including efflux pump overexpression (323). In other cases, such as in *Vibrio*, a direct interaction has been suggested between the beta-lactam agent and a sensor histidine kinase, leading to the induction of beta-lactamase production (370).

Two-component regulatory systems (TCS) are involved in a number of antibiotic resistance-inducing processes, such as VanA-operon-mediated vancomycin resistance, which involves the VanS protein detecting the signal produced by glycopeptide action, thereby activating (phosphorylating) VanR, acting on the essential promoter of the Van operon (371). The TCS-mediated processes (and the intensity of induction) might be

modulated by other proteins, termed TCS connectors, by affecting the phosphorylation state of the response regulators (372). Most if not all inducible mechanisms leading to antibiotic resistance have evolved in the absence of antibiotics, and therefore the induction mechanism should have physiological and regulatory functions. For instance, the *erm* gene family encodes inducible resistance to macrolides, lincosamides, and streptogramin (MLS) antibiotics by producing enzymes that catalyze S-adenosyl-L-methionine-dependent methylation, an adenine residue in the 23S rRNA gene molecule, resulting in the loss of MLS binding to the ribosome. The induction mechanism, provoked by ribosome stalling, involves a change in the hairpin secondary structures of mRNA, allowing the expression of the methylase. This mRNA attenuation mechanism is found not only in antibiotic producers but also in many Gram-positive organisms (102), most of which are non-pathogenic, and *Bacteroides* (362), which suggests that induction evolution is related to bacterial growth physiology, as regulation of protein synthesis and protein folding (373). Some of these mechanisms (non-*erm* related) are weakly induced by MLS antibiotics, resulting in low-level resistance (374).

The inducer of the resistance mechanism might not be the antibiotic but rather the change in concentration of a physiological substance, which might occur due not only to the antibiotic's action (as an accumulation after a pathway has been disturbed by antibiotics), but also as a consequence of physiological processes regulating the pathway. If the endogenous substance is increased for any reason at a given time, antibiotic resistance will increase, even in the absence of antibiotics. In addition to endogenous inducers, ARGs can be induced by exogenous compounds, which is the case for efflux pumps that serve to adapt bacteria to the potential injuries present in its habitat (375), that responds to bile, present in the gut of the colonized host (376) and efflux pumps from

environmental pathogens, such as *Stenotrophomonas maltophilia*, whose expression is induced by plant-produced compounds (104).

In general, the mechanisms for the evolution of inducibility are thought to be based on the coordination of economy (fitness), preventing the production (and its consequent cost) of traits that have no function except in the presence of the substrate, and preventing the deleterious dysregulation associated with production "at an inappropriate time" in the cell's physiology. The net result is the plasticity of bacterial behavior when confronted with changing environments, with different possible outcomes (377). However, if exposure to the challenging agent is frequent, a constitutive (constant) expression will spare the costs related to the induction machinery processes, the "costs of phenotypic plasticity" (378, 379). Depending on the frequency of the exposure to the challenge (the antibiotic), either induction or constitutive (constant) expression of the mechanism can evolve. Both alternatives are not necessarily orthogonal, and there are some cases in which they might evolve in parallel (380). For instance, rapid bacterial killing by the antibiotic might prevent the survival of inducible cells before induction takes place. These cases include those involving the constitutive production of an antibiotic-inactivating enzyme released into the environment (such as a beta-lactamase) by a relatively small fraction of the bacterial population, acting as "cooperators" able to protect the majority of "bacterial cheaters" in close spatially-structured populations (381). This production acts as in the common good, reducing the local activity of the drug and facilitating the survival of many inducible cells in the population during early exposure, which are then induced and reach full resistance. The proportion of "constitutive" resistant cooperative mutants for a particular gene (mutation rate plasticity), in relation to the cheater inducible population, might reflect these global adaptive needs.

The bacterial population size (and density) in a given compartment should be a predictor of the local availability of mutants favoring constitutive expression from inducible genes and is a neighborhood marker, favoring "the common good". Consequently, cross-signaling can be expected between quorum-sensing mechanisms and bacterial mutations or inducibility of antibiotic resistance mechanisms. Efflux pumps extruding antibiotics from environmental pathogens such as *Stenotrophomonas maltophilia*, whose expression is induced by plant-produced compounds is an example of it (104). However, there is little evidence for such a putative relationship. Maybe quorum-sensing modify the rate at which a bacterial population mutate to antibiotic resistance depending on their biological environment (382, 383). On the other hand, exposure to low antibiotic concentrations results in the selection of quorum-sensing-negative *S. aureus* (384). More recently, Hernando-Amado et al. stated that the evolution of antibiotic resistance is contingent on the quorum sensing network (184). In general, increases in population density and size might well influence the variation and fitness effects of mutations (385).

The evolution of antibiotic-inducible resistance should mirror the costs of constitutive resistance. The resistance mechanisms involving several genes (386), major epigenetic constraints, or complex high-cost molecules will likely be more prone to inducibility. There are intermediate solutions such as the "weak constitutive production of the resistance mechanism", or "unspecific inducibility systems". Among the latter, inducibility of global stress responses to unspecific unidentified attacks might influence the early survival of specifically inducible organisms. The hypothesis that highly produced protein molecules are more prone to misfolding and could decrease fitness has not been confirmed (387).

In summary, antibiotic resistance that imposes high costs in most cases does not appear to evolve toward an inducible regulation. If the regulation does occur, however, it is

because the “physiological inducibility” of the genes involved in the processes is affected by the antibiotics. The expression of certain resistance genes can also be regulated via an antibiotic-responsive ribosome-mediated transcriptional attenuation mechanism (388). The role of the “regulatory genome” is probably critical to understanding the evolution of antibiotic resistance (389).

Selection of persistence and the evolution of antibiotic resistance. The conceptual differences between resistance, tolerance, and persistence have been analyzed in depth (390). In resistance and tolerance, the entire bacterial population is involved. Persistence is a property of a fraction of an otherwise genetically susceptible bacterial population that exhibits phenotypic insusceptibility (persistence) to antibiotics, being able to survive (viable) in the presence of antibiotics at concentrations in which the majority of the population is dies off. Persistence is spontaneously reversible (noninheritable), such that cells regrown from these refractory bacteria remain as fully susceptible to the antibiotic as the original population (391, 392). Stress favors the switch to persistence, which is frequently related to the random induction of alarmone (p)ppGpp activation (393). Mechanisms involving the sensing of the early damaging effects of antibiotics by two-component regulatory systems (394) or the processing of misfolded proteins (395) are also likely involved. However, the persistent subpopulation resulting from such a reversible switch can be selected during antibiotic exposure (396). Moreover, the persister phenotype frequently offers protection from death from a broad-spectrum of unrelated antimicrobial agents (cross-tolerance). The evolutionary importance of this type of “phenotypic selection” is that it might facilitate the generation and ascent of inherited, specific resistance to antibiotics (397), including antibiotic combinations (398), or it might promote the spread of antibiotic resistance plasmids (236). The mechanisms leading to this phenotype-to-genotype transition might involve both the generation of

variation and selective processes. On one hand, stress-response programs involved in the generation of persistence might also accelerate genome-wide mutagenesis and horizontal gene transfer (399–401). Persistence ensures viability and hence the ability to evolve but does not necessarily indicate the total absence of antibiotic effects on the cell Thus, persister variants able to acquire certain replicative abilities in the presence of the antibiotic should be selected with their heritable changes. In summary, there is an epistatic synergistic interaction between resistance and tolerance mutations that has been experimentally observed in strains evolved under intermittent antibiotic treatment (402).

An important issue in this respect is how antibiotic resistance evolves in nongrowing populations. The nongrowing status is frequently a phenotypic adaptation to different types of bacterial stress, most mediated by the stringent (p)ppGpp-RpoS response, in reaction to not only nutrient starvation (including low levels of carbon, nitrogen, or phosphorus) but also oxidative, osmotic, and temperature stress (403) and most likely immune (phagocytosis) and antibiotic stress (404). Bacteriostatic drugs produce a nongrowing status that pushes the cellular machinery to the "style of life" under non-replicating conditions. Given that environmental conditions (including antibiotics and other stressors) induce the same set of responses involving similar regulators, all leading to a nonreplicating status, a general core hormetic (dose-dependent) stress response has been proposed (405). A nongrowing status might increase the mutation rate and thereby the selection of mutational traits under antibiotic exposure (404). In the adaptation to antibiotic exposure, there is a conflict between noninheritable antibiotic protection associated with nongrowth and the selection of genetic mutants, which is of particular relevance in antibiotics stopping the growth rate (such as ribosomal inhibitors and numerous others at subcidal concentrations). However, nongrowth is somewhat

heterogeneous in bacterial populations, providing an intermittent chance of evolving genetic resistance.

The evolution of antibiotic tolerance, either by increasing the drug concentration that the bacteria are able to tolerate or increasing the proportion of tolerant variants, is an interesting issue that has been scarcely investigated (406). The number of genes involved in bacterial tolerance (the tolerome) is larger than the number of genes identified for the resistome, suggesting that the evolution of increased tolerance might evolve even faster than antibiotic resistance (390). Consequently, the question is whether tolerance reduces resistance or favors survival under antibiotic (potentially mutagenic) exposure, thereby increasing antibiotic resistance.

Selection of Antibiotic Resistance

Antibiotic selective concentration gradients in time and space: concentration-dependent selection and multivariate landscapes. There is a correspondence between antibiotic concentrations and the selection of bacterial genetic variants with various levels of antibiotic resistance. Low-level antibiotic concentrations, including those subinhibitory concentrations reducing the bacterial growth rate to a certain extent, select for organisms with both low and high-level resistance (i.e., MIC values). High antibiotic concentrations select only for organisms with high resistance, because those with lower levels are inhibited or killed. However, the consequences of these selective forces might differ. The number of very low-level resistance mechanisms (many derived from gene mutations providing housekeeping functions) are only revealed at very low antibiotic concentrations, which increases the bottleneck to low-level/intermediate ranges and the number of genetic low-level/intermediate resistance mechanisms, which, in any case, are more numerous than those providing high-level resistance. With strong

bottlenecking, strong selection for a few mechanisms is expected to occur (246, 407, 408).

Subinhibitory antibiotic concentrations might increase cellular stress and the mutation rate. Thus, low-level antibiotic concentrations are expected to select numerous competing beneficial variants, likely preventing the effective selection of the more evolvable ones, those likely to increase their resistance levels. The evolvability of a particular bacterial lineage and the possibility of achieving fixation is greatly influenced by its coexisting competitors (409, 410). Low-level antibiotic exposure likely spares many susceptible cells, resulting in a low effective strength of selection. Low selective force might even compensate for the mutational consequences of stress-induced populations (411). Fewer resistant variants are therefore expected to emerge under exposure to high-level antibiotic concentrations; however, those variants would have high-level, highly specific resistance mechanisms.

In the real world, bacterial populations are exposed to antibiotic gradients, the consequence of the molecules' diffusion in a continuous space. When antibiotics are administered to a particular host (such as human patients and livestock), there is a wide set of gradients of antibiotic concentrations in the tissues and mucosal surfaces, and bacteria are subjected to a diversity of concentrations (412, 413). The release of antibiotics in natural ecosystems through wastewater further expands the range of antibiotic concentrations that bacteria can encounter (see below). Each concentration (each point in the gradient) should be able to inhibit the population susceptible to it and to select the organisms able to resist this concentration; however, further up the gradient, these organisms might be inhibited or killed. The selection of a particular variant therefore takes place only in a "window of selection". For instance, antibiotic concentration gradients allow for the selection of different bacterial mutants at different points on the

gradient, a process termed “concentration-dependent selection” (414, 415). Competition between variants might thereby be spared (416). Concentration gradients create “environmental spatial diversity” (265), which, when confronted with the “genetic diversity” of bacterial cells, enables the precise selection of particular variants with even small phenotypic differences, enabling a step-by-step evolution from low to high-level resistance, favored by gradient shifts (264, 412, 416, 417). In nature, fluids might force bacteria to favor or oppose gradients; convection into areas with higher antibiotic concentrations might increase the selection of resistant mutants (418). In particular environments (e.g., soil, soil-water currents) and physical structures (e.g., natural clays and microfibers) might alter the bacterial cell membranes and facilitate the acquisition of resistance (419).

Once the high-level resistance trait is acquired (particularly in nonstructured spaces where high antibiotic concentrations are frequently present), we can expect an increased invisibility of the mutations influencing the first adaptation to low antibiotic concentrations (420), which now become irrelevant. Therefore, their adaptive costs are minimized by back mutation or gene replacement. For cases in which this high-level resistance mechanism is unavailable and in the presence of not-too-steep gradients, a collection of low-level mechanisms might produce a high-level resistance phenotype, such as in the case of carbapenem resistance in *E. coli* (421). Low level mutational resistance to carbapenems (in outer-membrane proteins or in PBPs) facilitates the acquisition of a carbapenemase gene (422) .

However, in structured (compartmentalized) spaces, the release of high local antibiotic concentrations will necessarily produce a space with low concentrations, and selection for different resistant variants is expected to occur across a stable gradient. As a result of diffusion laws, the space covered by low antibiotic concentrations will be larger over time

and much larger than the space covered with high concentrations (265). Therefore, local antibiotic exposure in compartmentalized spaces might in fact produce a "**bunch selection**" effect, in which allelic variants of various levels of antibiotic resistance are selected as a group or cluster in neighboring spaces. This spatial proximity and the possible gradient fluctuations facilitate cross-recombination between independently selected variants. Even at very low antibiotic concentrations, stochastic clearance of bacterial populations might occur (423).

A relevant issue is the concentration at which the significant gradient for the antibiotic effect begins to act. This concentration will depend on the minimum selective concentration, which is much lower than the MIC (58). Selection will result from several pharmacodynamic functions, including the Hill function, which describes the shape of the bacterial growth dose-response curve (16, 424). This minimum selective concentration can be compared with the "minimal effective antibiotic concentration" (MEAC),, which is the minimum antibiotic concentration able to produce *any* effect on bacteria (e.g., by acting as a signal and by influencing metabolism) (363, 413).

Specific antibiotic concentrations along gradients might also act to induce the expression of resistance mechanisms, including chromosomal and possibly plasmid-mediated beta-lactamases (425). In other cases, such as in antibiotics acting on ribosomes, the drug's effect at certain antibiotic concentrations might produce alterations in complex gene regulation, leading to bistability, i.e., bifurcation of a genetically homogeneous population into two subpopulations of different phenotypes (susceptible and resistant), favoring the selection of the resistant one (426). The spotted selection by particular antibiotic concentrations is highly dependent on the variant growth rate (426), the antibiotic's pharmacodynamics (181, 427, 428), the therapeutic regimens (429), and possibly other host factors (430).

Antibiotic gradients not only vary over time but are frequently embedded in other variable gradients, due, for example, to the presence of other antimicrobials and other selective attractors, producing multivariate extended selection landscapes (431). In these cases, selection occurs because of an integrated (but heterogeneous) global selective force in which the selected effects of traits or evolutionary individuals respond to this need of "global fitness". Additive genetic variances and covariances of phenotypic traits shape this global fitness (432). The representation of these integrated multivariate landscapes is a challenge for determining evolutionary trajectories in antibiotic resistance.

The "wicked problem" of modern antibiotic resistance and the history of antimicrobial selective pressure. How has the history of antibiotic selection, based on the sequential discovery, use, and release of antimicrobial substances, influenced the evolution of antibiotic resistance? Evolutionary trajectories are historical events resulting from descents over time with modification at given times. Past events in a given historical moment will occasionally (but not necessarily) influence future events. Antimicrobial selective events modify the bacterial world, influencing the entire hierarchy of units of selection (61).

The early 20th century (1910–1945) saw profound physical and social changes, including troops mobilizations in two world wars, worker and refugee movements, the emergence and development of big pharma, intensive farming, extensive mining, and the growth of the food industry. During this period, the world also endured massive industrial pollution and ecosystem damage, with the colossal mass production and application of synthetic antimicrobial agents in humans and animals for prophylaxis/antiseptic/therapeutic purposes, a situation frequently ignored as a factor affecting the future evolution of antimicrobial resistance. Antibiotics had in fact been employed since the mid-1940s in human and animal medicine in the midst of a massive increase in the production of anti-

infectives (433–437). From the late 1910s to the late 1940s, a plethora of old and new antibiotic and antiseptic compounds were simultaneously and massively employed in crowded settings, such as troops in the military, livestock on farms, and patients in hospitals. By 1907, Paul Ehrlich had already identified how *Trypanosoma brucei* became resistant to the trypanocidal activity of pararosaniline (arsenic), one of the 605 compounds analyzed before developing Salvarsan in 1909 (438). However, the first example of antimicrobial resistance in bacteria dates back to 1924, when an arsphenamine-resistant strain of *Spirochaeta pallida* was documented in a clinic in Germany after prolonged use of arsenicals for treating syphilis (439). Similar observations were made in France and the US in subsequent years, and antibiotic policies began by cycling the antibiotics with other therapeutic options (such as mercury and bismuth salts), increasing the dosage when necessary (440). Sulfonamide resistance also emerged soon after the drug's commercial release in 1935, as reflected in reports on pathogens causing severe diseases, such as *Neisseria meningitidis*, *Streptococcus pyogenes*, *S. aureus* (441), and many other species after the end of World War II (442, 443). Penicillin resistance was documented in *S. aureus* in 1942 (444) and at the time was only demonstrated *in vitro* in streptococci (mutant selection) (442). The anthropogenic use of antimicrobials includes significantly heavy metals; in particular, copper and silver salts, which have been historically employed in treating surgical wounds, postpartum vaginal tears, and gonorrheal infections. Mercury and tellurite salts and arsenates have been employed to treat several infectious diseases. The organoarsenic compound arsphenamine (Salvarsan) was introduced in the 1910s and was the first effective treatment for syphilis, the starting point for chemotherapy. Copper and silver vessels have been employed for at least three thousand years to decontaminate water and food (445). The translucent, white, and colored glazes of ceramic vessels and kitchenware might also release antiseptic

concentrations of the lead, cadmium, chromium, and cobalt (446). Interestingly, many of the "modern" plasmids (and transposons) encoding antibiotic resistance contain determinants encoding for heavy metal resistance, leading to the speculation of whether the selection of these replicons predated the current antibiotic-resistant mobilome.

The history of modern antibiotics is even more relevant to our understanding of the evolution of significant resistance genes (1). Synthetic dyes and sulfonamides were subsequently introduced for treating infections, followed by penicillin, streptomycin, tetracycline, chloramphenicol, kanamycin, and neomycin (447, 448), all of which selected for organisms carrying genes able to detoxify the various antibiotic agents. In terms of the evolution of antibiotic resistance, the important fact is that these genes remain present, are mostly intact, and are still prevalent today, despite these older drugs being replaced by novel molecules that overcame the gene's resistance mechanisms. For instance, the same *sul* genes are currently present in integrons, despite sulfonamides not being widely employed. ARGs might eventually provide some adaptive advantages unrelated to antibiotic resistance, such as in the case of tetracycline resistance (449, 450). The reduced clinical use of particular antimicrobial agents does not ensure a reduction in the prevalence of resistance genes (451). There are several explanations for the persistence of resistant phenotypes in bacterial populations (452, 453), including the periodic replacement with resistant clones (454). This apparent "bacteria never forget" behavior facilitates the evolution of multiresistance by genetic capitalism, the concept that resistant bacteria tend to be increasingly resistant. (see Section 4.4.3 Genetic capitalism,).

As to whether there are particular antibiotics or groups of antibiotics more prone to pushing evolution to higher resistance, we should first consider that antibiotics whose resistance genes are present in widespread mobile genetic elements contribute to the

selection of these transmissible units, eventually carrying other resistance genes. Plasmids containing *bla*TEM-1 are ubiquitous in bacterial pathogens; the overuse of aminopenicillins might have contributed to the recruitment in these plasmids of genes encoding resistance to third-generation cephalosporins. Second, antibiotics select resistance genes or their variants, promoting high MICs in themselves and other related drugs, such as ceftazidime and CTX-M and VIM beta-lactamases (see later).

Genetic capitalism: resistance traits, global fitness, and evolvability tools. The term "genetic capitalism" in antibiotic resistance refers to the capability of organisms to accumulate resistance mechanisms, either via mutational or gene acquisition events, such that the acquisition of a resistance trait facilitates the acquisition of further resistances — the rich tend to become richer (60). This concept can be illustrated in the recent known cases of MRSA, multiresistant pneumococci, vancomycin-resistant enterococci, extended-spectrum beta-lactamase, and carbapenemase-producing Enterobacterales. Genetic capitalism enlarges the field of selection (through multilateral antibiotic selection) under antibiotic exposure and has likely influenced the increased prevalence of MDR pathogens and the spread and maintenance of resistance genes among environmental organisms and commensal bacteria, including those of normal microbiota.

Genetic capitalism might work without antibiotic exposure. Organisms with mutations leading to reduced antibiotic susceptibility frequently emerge during the process of adaptation to particular growth conditions (152). Adaptation to environmental changes generally tends to increase the number of enriched bacteria in mutational traits (or the acquisition of foreign genes), which might facilitate antibiotic resistance. Similarly, bacteria under antibiotic exposure or under general situations of stress or adaptive need can be enriched by evolvability tools, e.g., the acquisition of mobile genetic elements

(from plasmids to insertion sequences), which might serve as sculptors of antibiotic resistance complex determinants.

Pharmacodynamics and selection of antibiotic resistance. Do the bactericidal or bacteriostatic effects of antibiotics have any influence on the frequency, spread, and evolution of antibiotic resistance? Many ARGs in natural populations correspond to bacteriostatic antibiotics, such as tetracycline, chloramphenicol, macrolide-lincosamide, and sulfonamide. Among the bactericidal antimicrobials, only genes detoxifying compounds acting directly on physical cell structures (rather than processes) appear to be less prone to contributing to the emergence or selection of resistance genes. For instance, the evolution of resistance to antimicrobial peptides, including those involved in a human or animal host's innate immunity, appears to be scarcely effective (455). The differentiation of antibiotics into bacteriostatic and bactericidal is extremely dependent on human criteria. In addition to pharmacokinetics (available antibiotic in contact with the bacterial cell), numerous factors modulate the cidality of antibiotics, such as cellular responses, the expression of SOS and RpoS systems, the effect of reactive oxygen species, and metabolic and environmentally regulated adaptations (456). Hypothetically, less cidal antibiotics could preserve susceptible populations more than stronger cidal antibiotics; however, high cidality should reduce the cell's possibilities of adapting to the antibiotic challenge. In addition, many bactericidal antibiotics are bacteriostatic at low concentrations, as those that are expected to occur in the long tale of gradients, both in treated patients and in environmental settings. Acquired antibiotic resistance mechanisms are as apparently equally numerous for bacteriostatic and bactericidal antimicrobial agents.

Strategies of antibiotic use and evolution of resistance: collateral sensitivity, collateral damage. Antibiotic resistance is correlated with antibiotic exposure (457,

458). A number of mathematical models suggest that reducing the rate at which individuals are administered antibiotics is more effective than reducing the treatment duration (459). The dominant interventions for changing the threatening landscape of the emergence and spread of antibiotic resistance include strategies for antibiotic use. Interventions against the emergence and early evolution of resistance have particular interest for individual patients. Once resistance has occurred, however, preventing the "spread" becomes the main target for protecting society from resistance. Combination therapy has demonstrated efficacy among the successful methods for employing antibiotics to prevent emergence and early evolution. The alternating use of drugs (in which different drugs are cycled during treatment) has been shown to slow evolution by constraining the mutational paths toward significant resistance (460). However, this strategy is less effective than the simultaneous administration of drugs, such as in bitherapy and multitherapy (461). A promising complex approach to decelerating resistance evolution in controlled evolution experiments is the sequential use of pairs of antibiotics, particularly when the resistant bacteria present collateral sensibility, as discussed below (324, 462).

In particular, the strategies for employing drugs in closed environments (e.g., farms, hospitals, and long-term care facilities) have been theoretically and experimentally evaluated. The "crop rotation strategy" (463) is similar to the alternating drug use in individual patients, cycling various types of drugs in a patient group (e.g., in the ICU). However, theoretical and *in vitro* "cycling" models are unlikely to reduce either the evolution or spread of antibiotic resistance. The other option is "mixing", in which different patients are treated with various types of drugs that might be more effective. (464, 465). The timing of the "cycling time schedule" (when the second drug replaces the first) might be critical; rapid replacements or even random replacements might be more

2002 effective than conservative switches (466). The adaptation of prescriptions and
2003 therapeutic schedules to the local resistance landscape (provided by surveillance studies)
2004 could be effective (467). The treatment of all patients with a combination of antibiotics is
2005 in most cases the optimal treatment strategy both for the patient and the group (465).
2006 "Multiday cycling" with antibiotic combinations based on collateral sensitivity has shown
2007 promise in mathematical models (468).

2008 In terms of the combined or successive use of antimicrobial agents, the field of collateral
2009 interactions (the effects of one antibiotic modifying the effects of others) has attracted
2010 interest over the last decade. Mutations that were shown to cause MDR in bacteria
2011 simultaneously enhanced sensitivity to many other unrelated drugs (collateral sensitivity)
2012 (469–471). These effects are essential to understanding the evolution of resistance to beta-
2013 lactams plus beta-lactamase inhibitors (472, 473). Different collateral effects (collateral
2014 sensitivity and cross-resistance) have been shown to evolve in parallel experimental
2015 replicate *P. aeruginosa* populations subjected to beta-lactams and aminoglycosides,
2016 frequently by mutations in regulatory genes (474). Interactions between the effects caused
2017 by several combined drugs might favor suppressive effects ("more is less") over
2018 beneficial ones such as enhanced killing (475). Antibiotic-resistant bacteria tend to
2019 increase their sensitivity to antimicrobial peptides (476), including colistin and
2020 polymyxin (477).

2021 **Resistance dynamics in the presence of diverse antimicrobial agents and**
2022 **antiresistance strategies.** The evolution of antibiotic resistance frequently occurs in the
2023 simultaneous or fluctuating presence of several antibiotics in the same host or
2024 environment. Experimental evolution studies have revealed that lineages exposed to
2025 combinations of different antibiotics evolve a different allele dynamic than in the case of
2026 exposure to a single drug (478). Mutations without a resistance phenotype might

modulate the activity of a resistance enzyme to facilitate activity to two different antibiotics (479, 480),. Many of these phenomena are explained by the phenomenon of antagonistic pleiotropy or collateral sensitivity (see above). The resistance dynamics in the presence of different antibiotics is also influenced by the drugs' effects on the host's microbiota, creating "opportunities for the colonization" of resistant variants of each single drug. In general and particularly in high-risk epidemiological situations, however, there is an clear need for associating all available resources to limit the spread of resistance, "breaking barriers" among antibacterial compounds (including antiseptics) and strategies (481).

THE ECOLOGY AND TOPOLOGY OF EVOLUTIONARY TRAJECTORIES OF ANTIBIOTIC RESISTANCE

Trajectories and Fitness Landscapes of Antibiotic Resistance

In the classic fitness landscape metaphor (Figure 4) developed by Wright (482), which essentially persists in modern computer-generated landscapes, there is a "horizontal plane" (with different genotypes represented by binary sequences of two types of basic units) and a network of possible mutations between the genotypes forming a hypercube graph. The fitness (reproductive success) of each of these genotypes is represented by a corresponding "height" on the vertical axis. In this plane, the binary (0/1) representation shows the absence or presence of two different alleles of a gene or a particular point mutation. Other "beyond the hypercube" computer landscapes, considering not only binary representations but also 4 (nucleotides) or 20 (amino acids) alternatives, might produce more realistic landscapes, with higher possibilities of finding trajectories to gain access to fitness peaks (483). How many genotype possibilities are contained in this "soil"

plane? In terms of nucleotides, one of the organisms with the smallest genome, the Proteobacteria *Nasuia deltocephalinicola* (112,091 nucleotides) can reach 10^{67430} genotypes (483).

Natural selection forces populations to follow evolutionary trajectories along uphill steps of increasing fitness (482, 484). The important issue in the predictability of evolutionary trajectories is when there is only a limited number of trajectories available, travelling from distinct adaptive peaks to reach a final optimal genotypic state (485). (Figure 4)

In multipeaked fitness landscapes, as in real environments that might be highly variable both in space and time, evolutionary trajectories necessarily should be able to cross valleys, with low fitness and a certain risk of stasis or extinction of the evolutionary objects. It is widely assumed that many if not most adaptations are associated with trade-offs, such that changes in traits that increase fitness in some environments or situations are deleterious in other environments or situations (486). For instance, a resistance gene can help the host strain climb high fitness peaks during therapy. In the absence of antibiotic exposure, however, this gene might lead the organism into a valley, resulting from a gene burden for the cell physiology. The changing dynamics of fitness landscapes constitute the main condition of evolutionary changes. Occasionally, the mutation providing access to the most efficient fitness peak in terms of antibiotic resistance is suboptimal for metabolic activities, and the best mutant is the one that climbs an intermediate fitness peak for resistance, maintaining the most metabolic-based fitness (487).

In some cases, survival in valleys might facilitate climbing the next fitness hill. Initially deleterious mutations (sinking the strain in the valley) might serve as gateways for otherwise relatively inaccessible areas of sequence spaces, which might result in positive epistasis with other mutations, thus facilitating uphill trajectories, as observed with TEM-

15 beta-lactamase (488). As recognized by Sewall Wright (489), epistasis can also cause the fitness landscape to possess ridges and valleys that constrain the ability of evolving populations to reach the genotype of highest fitness. For instance, antagonistic interactions are not infrequent and tend to decelerate the pace of adaptation (490). "Long-term advantageous" but at first sight deleterious mutations can be fixed in small populations, and even slightly deleterious ones can also be fixed in relatively large populations (491, 492).

Given these potential advantages, sufficiently large bacterial populations can cross fitness valleys, which is probably not the case for small populations (493, 494). Probably but easy-to-reach but small population variants located in valleys have only a small chance of finding small "peaks" scattered inside the valley. Larger populations, however, might attempt to scale the slopes of higher fitness peaks. It is possible that competition might occur between simple and complex evolutionary trajectories. In rugged landscapes, simple trajectories tend to exploit the immediate easy-to-reach fitness peak. In doing so, however, access to higher peaks might be hampered. In the presence of high population sizes, the fixation of beneficial mutations takes longer, and the genetic diversity of the population is maintained, favoring the collection of adaptive mutants and their interaction, potentiating the population to climb higher peaks by "stochastic tunneling" (495, 496). In any case, we stress here the importance of "abundance" in the evolution of antibiotic resistance the organisms presenting greater population abundance have a greater chance of finding effective evolutionary paths to increased resistance.

Trajectories and Flows in Free-Energy Fitness Landscapes

The biological local optima (higher fitness) are frequently represented as peaks on the fitness landscape, a powerful metaphor (albeit an anthropocentric view, given that our

evolutionary units are not subject to gravity) indicating that climbing peaks represents success. However, fitness landscapes are not always depicted this way. The fitness function corresponds to the concept of a potential or energy function in physics, in contrast to the conventional representations in physics and physical chemistry, including protein and RNA evolution: higher fitness is instead associated with lower altitude on sequence-space landscapes (497–499). The rationale has a thermodynamic base: the most stable (high fitness) configurations are those associated with the lowest free energy local minimums (500). As stated early in this review, evolution is a stress-reducing trend. The lowest free energy can correlate with the lowest stress. The relationship between stress and changes in entropy (stress entropic load) has been discussed previously (501). In this type of "inverted fitness landscape", valleys describe evolutionary trajectories leading to increased fitness, and even funnels in the soil of the valley might direct the trajectory to profound fitness. The advantage of this representation is that it helps picture adaptive trajectories as flows, where, as in nature, the density of the flowing units helps overcome obstacles through the higher-fitness basins of attraction. These obstacles in fact correspond to the "evolutionary constraints" shaping the evolutionary trajectories.

Evolutionary Trajectories in Crumpled Landscapes

The standard bidimensional and tridimensional representations of fitness landscapes have contributed to the understanding of evolutionary trajectories. However, these representations are insufficient for imagining extremely complex trajectories crossing deep fitness valleys and spaces when the fitness peaks are spaced far apart. However, imagine smoothing out the creases caused by crumpling a sheet of paper into a ball. The result is a wrinkled texture with "peaks" and "basins" formed by the confluence of creases, which resemble a fitness landscape. These irregularities were (probably more pronounced) in the paper sheet before the ball structure was disturbed. However, the

fitness peaks that are distant from each other in the smoothed-out state can be spatially close in the crumpled form (Figures 4 and 5), meaning that a particular genotype has access to increased fitness in another peak apparently inaccessible in a flat landscape. The number of adaptive fitness peaks is proportional to the number of genotypes analyzed (which is higher than the binary traits), as is the case with computer-generated fitness landscapes, and to the number of selective forces present in a particular landscape.

In general, fitness landscapes deal with adaptation to a single need (e.g., a certain level of resistance to a particular antibiotic). Varying antibiotic concentrations across a gradient might produce multiple peaks because of a concentration-dependent selection of genotypes (414). In nature, genotypes are challenged by a diversity of adaptive needs located in the "same landscape",, so that fitness points across the landscape are represented by multiple peaks, sometimes combined peaks, determining accessible evolutionary paths (502). These multimodal peaks frequently produce a rugged landscape where the "ecology" of various genotypes are represented. From the reductionism imposed by the scientific method, there are areas in the real world with a high consumption and/or high heterogeneity of antimicrobials at various concentrations, with different types of hosts with different microbiomes. The resulting fitness landscape should have more adaptive peaks and deleterious basins, and the "crumpled ball" should better reflect the possibility of a particular genotype's access to higher combined fitness for different needs (503). There are no "Darwinian demons" able to reach high fitness in all environments (504), but the emergence of high-risk bacterial genotypes combining multiresistance, virulence, colonization, and epidemigenicity might result from the confluence of fitness peaks. Complex environments that are more demanding and stressful should produce more peaks and basins, which can be represented by the compressing, squeezing intensity exerted on the crumpled paper ball. Despite the high

complexity of the resulting landscape, this "intensity" might be measured by a single global quantity. The evolution of damage in crumpling dynamics can largely be described by a single global quantity: the total length of creases (505). The physics and complexity of crumpled balls have been studied (506) but not its evolutionary applications.

Genotype by Environment Interactions: Environmental Merging and Coalescence of Microbiotas.

A high frequency or random changes in the bacterial genome have consequences on the fitness of bacteria in different environments. In a classic study, individual random insertion mutants of *E. coli* were assayed in four different environments and found that approximately 40% of the insertions yielded different fitness effects in the different environments, showing that genotype-by-environment interactions are common (507). There are environment-specific mutational fates; ligand binding, a mutant enzyme, or protein stability can result in differing bacterial fitness across environments (508). Different environments (e.g., water bodies, farms, grassland, forest soil, the inside and surface of animals, and hospitalized patients) have different resistomes, and the evolutionary paths toward significant resistance can differ significantly (51). An essential goal of research in antimicrobial resistance is to quantify the risks for antibiotic resistance of environmental overlapping (136, 509, 510).

Merging resistome-rich environments provides a wealth of possible new operative material (genes), vehicles (such as mobile genetic elements), and genetic partners, able to produce unexpected evolutionary trajectories. The strong cross-environment mobility of ARGs has been documented (78). Genes from the environmental resistome (such as SHV beta-lactamases) have intertwined evolutionary histories with those of clinical origin (511). It is essential to understand and control the situations in which humans and

particular high-risk animals have an interactive ecology (including food), particularly for multihost pathogens (512, 513).

The coalescence of microbiota from humans, animals, and environments, and its possible effect on the spread and evolution of antibiotic resistance has recently been reviewed (514). Microbiome merging (515) has been facilitated by recent world globalization, with deep sociodemographic and dietary changes in human populations; particularly, by a high density of food animals with their microbiomes (there are currently approximately 23 billion chickens and 770 million pigs in the world). There has also been a strong decline in animal diversity, which can be attributed to the human (artificial) selection of a small range of animal varieties of economic interest. Microbiota might therefore circulate among almost identical animals without the ancient constraints imposed by the different animal varieties (514). In poorly sanitized regions in particular, resistance genes can spread through untreated wastewater, antibiotic exposure can result from treating human infections, and antibiotics are employed for farming purposes, all of affect the abundance of ARGs in environments where animals can acquire "human microbiota communities" (see below). Human fecal pollution can be traced by detecting sequences of human-microbiota-specific phages, such as the crAssphage (516). Conversely, close contact with farm animals might also modify the human microbiome (517). As we will discuss later, coalescence of microbiotas ensures the wide circulation of mobile genetic elements, providing opportunities for the spread of the mobile resistome (513).

The Role of Environmental Heterogeneity

All elements that affect the evolution of antibiotic resistance (e.g., genetic and protein sequences, genes, proteins, protein complexes, mobile genetic elements, clones, species, bacterial communities) are located in spaces. Their position in space will determine their interactive network and consequently their possible evolutionary trajectories (518). The

2200 interactive networks between evolutionary units involved in antibiotic resistance are
2201 frequently described as having a sociobiological nature and are sometimes modeled with
2202 game theory tools (519).

2203 Sociobiology depends on neighborhood, the relative position of the elements in space.
2204 The influence of "positioning" in bacterial evolution has been well documented in the
2205 case of dense, surface-attached, spatially structured bacterial communities (520).
2206 Selection of particular variants will occur at some "positions" in the space and not in
2207 others. Genetic variants might self-organize in the space, producing an adaptive radiation
2208 to find neighbor niches (521), eventually leading to a functional division of labor (522).
2209 This is expected to also occur in long-term batch cultures (where there is no passage of
2210 cells), in which the bacterial effects increase the heterogeneity of the environment,
2211 resulting in a multiple adaptation with coexistence of different variants (523)). The rates
2212 of environmental fluctuation might modulate the level of radiation in novel niches, and
2213 competition between variants and the benefits of the "ancestor niche" might act as an
2214 attractor limiting diversification (205).

2215 The importance of positioning appears clear in subcellular molecular topology. The
2216 evolution of proteins involved in antibiotic resistance depends on their location inside the
2217 cell, their intracellular and pericellular diffusion, and the local random obstacle networks
2218 (524). Recent studies in "contact genomics" suggest that DNA levels, the local possibility
2219 of collisions between segments of DNA molecules (including plasmid-plasmid and
2220 plasmid-chromosome interactions) are critical to shaping evolutionary steps and hence
2221 trajectories (525, 526).

2222 The case of plasmid interactions is indeed essential in antibiotic resistance, given that
2223 resistance genes use plasmids as vehicles to spread across bacterial populations. The
2224 sociobiological evolution of plasmid interactions to become co-resident in the same strain

by regulating their replication strategies and their copy number (527) is a major factor in resistance gene promiscuity.

Regarding particular bacterial populations and communities, the metacommunity framework indicates that local co-residence, facilitating the genetic exchange of antibiotic resistance, depends on the outcome of local species interactions and migrations. Local species' coexistence and exclusion within the multiscale and multispecies context within meta-communities should necessarily influence the evolution of antibiotic resistance, which will occur in spatially close colonization areas. In general, coexistence in joint ecological-evolutionary models requires low to intermediate dispersal rates that can promote the maintenance of both regional species and genetic diversity (528). Physical interactions are favored when particular organisms are located in the same niche-neighborhood (or share subniches in a single niche) and in close neutral spaces (e.g., niches in the mucosal intestinal membranes and neutral areas in fecal content). With weak dispersal separation, both neutral and niche-based interactions are mutually amplified (529, 530). Migration should increase the impact of the horizontal transfer of resistance, which would be limited in areas of replication, where vertical transfer predominates (531).

The consideration of environment variability in bacterial evolution is illustrated in the source-sink dynamics theory. A bacterial population can find an optimal patch in the environment in which to replicate, a patch that is then converted into a *source* of organisms. In the spatial vicinity of this source patch, there can be population-free patches that scarcely allow for growth or even lead to a negative growth rate. These areas are known as sink patches. Given the population density in the source, a number of individuals are forced to move (migrate) from the source to the sink, which can be occupied even without facilitating growth. However, if we consider a more complex

landscape (such as the one created by the presence of two antibiotics), the source patch for resistance to one antibiotic (where resistant bacteria proliferate selectively) might eventually be a sink patch for another one, typically when antagonistic pleiotropy occurs (resistance to one antibiotic means more susceptibility to the other). Under these circumstances, the frequency of migration favors the evolutionary speed of antibiotic resistance minimizing the costs of adaptation (461).

Even considering a single drug present at different variable concentrations in a gradient or neighboring spaces, source patches might be able to produce sink patch colonization (532). The fitness variability of the environment frequently changes, albeit slowly, producing a "moving optimum" (533). The graduality of the changes might have different evolutionary consequences (534), particularly influencing populations with standing genetic variation; for instance, faster environmental change favors fixation of multiple alleles of small effect (535).

A theoretical framework for these evolutionary predictions with variable fitness peaks of antibiotic resistance was provided by Fisher's geometrical model, which helps analyze the contribution of several selectable traits to the high-fitness phenotype (536–538).

Ecologically Cohesive Populations and Genetic Exchange Communities.

Studies has recently and dramatically proposed that genes and not species inhabit niches; hence, ecologically adapted species (or populations) simply do not exist (531). This Dawkinian statement (the selfish gene) does not rule out the fact that genetic interactions require interactions between vehicles (cells and cell populations) that are efficient units of selection more than simple gene carriers. Interactive lateral genetic transfer between bacterial populations and communities (539) is required to establish many genetic evolutionary spaces. Thus, the study of the ecology of evolutionary trajectories

necessarily requires the understanding of the ecological cohesion between bacterial populations.

This important topic is studied by investigating the spatial heterogeneity and co-occurrence patterns of microorganisms in their habitats, including the human mucosal-associated populations (540, 541). Modern metagenomic-bioinformatic techniques, such as high-throughput chromosome conformation capture (3C) technology, might be useful for detecting ensembles if resistance genes hosted by particular bacterial species or groups of species (525, 526) can identify genetic exchange communities.

Why are groups of microorganisms spatially linked? We have discussed the above coexistence through the sharing of subniches; however, this implies a frequent "sharing of a common goal" (cooperation). Coexistence ensures a number of functional possibilities, eventually influencing the host's physiology. However, spatial linkage can also be due to negative interactions among groups of organisms (amensalism, competition) and with the host's local conditions, mostly the eco-active local chemosphere (141). In any case, organismal spatial linkage influences the resilience of local communities (109), and the method and rules by which bacteria associate (contact) in the space and their ecological consequences are insufficiently known (542).

Antibiotic Resistance in Minority Populations

Although ARGs can be found almost anywhere, the population of antibiotic-resistant bacteria that are relevant to human health are in the minority, and the number of resistance genes they have acquired is minimal, considering the large number of potential resistance genes present in nature. Various bottlenecks can modulate the acquisition of resistance. The first is ecological connectivity; although genes are shared by bacterial populations, the organisms receiving them belong to gene-exchange communities, usually formed by

bacteria able to form stable microbiomes sharing similar ecosystems. A second bottleneck to consider is the founder effect. Once a resistance gene is established in a population, the rewards for recruiting a new one with similar effectivity for counteracting antibiotic action will be minimal. Lastly, fitness costs will be fundamental for selecting those genes that impose a lower physiological burden when expressed in the new host (543).

Host-Environment Equilibrium as an Evolutionary Constraint: Evolutionarily Stable Strategies

An evolutionarily stable strategy is one that, if adopted by a population in a given environment, cannot be invaded by any initially rare alternative strategy. The term "evolutionarily stable strategy" comes from Maynard Smith's game theory, rooted in Hamilton's proposal of unbeatable strategy (544), meaning that a biological population permanently chooses not to take a risk for a benefit over competitors, ensuring in exchange a comfortable biological position. The unbeatable strategy occurs because the population is kept in a successful adaptive configuration (the strategy) ensuring an equilibrium with the environment, resulting in ecogenetic stability. This population might have possibilities for acquiring more effective traits leading to higher fitness (for instance, higher antibiotic resistance), but such acquisition implies possible conflicts with other adaptive traits of proven success (545). The minority variants proposing an alternative strategy are prevented from successfully invading the population. In other words, a minority population endowed with an evolutionarily stable strategy might have difficulty selecting successful resistant variants, including the acquisition of foreign chromosomal genes, which could be interpreted as "divergence hitchhiking", where, in which the possibility of diverging variants are prevented as a collateral effect of strong divergent selection on genes involved in local adaptation (342).

The Eco-Evolutionary Spaces of Gene Variation: Chromosomal Genes versus Mobile Genetic Element Genes

Gene evolution can be in turn considered a numbers game, depending on the number of gene copies, the gene's long-term stability, the diversity of environments to which the replicon hosting the gene is exposed, and the bacterial host and niche in which it is present. The number of gene copies (such as a preresistance or resistance gene) determines its evolvability rate, a number that primarily derives from the rate of host replicon replication (bacterial host, mobile genetic element) so that genes from the more abundant and spreading organisms should evolve faster. Given that genes in plasmids multiply in the host cell (304) and, taking advantage of the host replication, might propagate in different hosts (exposed to an expanded variety of environments), it can be expected that plasmid-located genes (including antibiotic resistance) should evolve more rapidly than chromosomal genes. (Figure 2).

Mobile genetic elements have another advantage for hosting rapidly evolving genes. The adaptive strategy of chromosomal variation (for instance, in genes encoding the targets of antibiotics) to increase antibiotic resistance might be considered much riskier in terms of fitness reduction for the bacterial host than for acquiring novel traits by mobile genetic elements. Chromosomal genes are frequently inserted into highly regulated interactive biochemical networks that cannot be modified without harm to the system's equilibrium. In addition, the functionality of heterologous chromosomal genes in a particular host is constrained by the compatibility with the host cell's physiology (546). In contrast, foreign genes acquired by horizontal gene transfer, such as ARGs, should in principle be better tolerated, given they are frequently "decontextualized"; the genes do not belong to the basic network involved in the new host physiology.

Various mechanisms of resistance are accessible by the evolutionary (mutational, recombinational) space of single organisms, such as SHV-type beta-lactamases in *Klebsiella pneumoniae*, which are very close to (and probably originated in) the chromosomal beta-lactamase proteins of this organism (547); however, the beta-lactamases probably only evolved when these SHV enzymes were propagated in plasmids. Certain highly efficient mechanisms of resistance are simply unavailable through chromosomal evolution in the original pathogenic hosts. CTX-M enzymes have not evolved in their original host (*Kluyvera* spp.); the only possibility of acquiring these characteristics has been by horizontal gene transfer when present in *E. coli.* The association between CTX-M encoding genes with successful widespread mobile genetic elements and bacterial clones (548, 549) and the optimization of their combinations have contributed to the explosive diversification of CTX-M enzymes. Expanding plasmid-host range by positive epistasis mechanisms improving plasmid persistence and spread have important implications in the spread and evolution of antibiotic resistance (550, 551).

Most importantly, a gene in multicopy (located in a multicopy plasmid) facilitates the acquisition of a new antibiotic resistance phenotype compared with the same gene when present in the monocopy (chromosomal location) (303, 552).

EVOLUTIONARY TRAJECTORIES OF ANTIBIOTIC RESISTANCE GENES

The Gene Space of Variation

The gene mutational space. The evolution of most ARGs is the evolution of particular changes in gene sequences, resulting in amino acid changes that increase or expand the host organism's fitness when exposed to antimicrobial agents. It is difficult to separate the "resistance gene mutational space" from the "resistance protein space of variation",

but; however, a correspondence between regions of the resistance gene sequences and the protein sequence spaces is expected (553). Mapping protein sequence space is a complex issue, given that for a protein of length N, the number of amino-acid combinations is 20^N. Mutational changes tolerated by the bacterial organism, however, might not necessarily produce a higher fitness phenotype; in many cases, mutations are neutral or "nearly neutral".

There are several possibilities. First, a single nucleotide variation giving rise to a synonymous codon should be effectively neutral, with no consequences for the protein's structure and function. Therefore, even if this variant could be enriched by drift, nothing will occur in terms of selective adaptation. Second, the nucleotide variation might produce an amino acid change influencing a protein domain but without phenotypic consequences and will therefore not be subjected to natural selection. The absence of expected phenotypic consequences (such as an increase in beta-lactam MIC) might not necessarily be interpreted by itself as full neutrality.

For instance, although the change in beta-lactamase conformation might not influence hydrolytic efficiency, it might affect the protein's stability and would therefore comprise a selectable change (554–557). Third, the nucleotide change might result in a protein change with all the appearances of neutrality (i.e., with no functional consequence), but the nucleotide change could influence the effects of other mutations that might occur later, either by increasing or reducing the possibility of natural selection (positive, negative, or sign epistasis (558). Fourth, the variant nucleotide might influence the phenotype but in an extremely subtle manner (such as producing tiny increases in MIC) resulting in the phenotype being overlooked by natural selection. This concept was proposed (for beta-lactamases) as "we do not know how small an effect constitutes a selective advantage"

(559). It has been shown, however, that very small phenotypic differences are indeed selectable across natural gradients (412, 414).

Take for example a space covered by all sequences of a gene connected by single-step mutation distances and providing an identical or almost identical phenotype to that provided by the most-fit sequence. This is a neutral or nearly neutral network. If this network is large then the protein produced by the resistance gene is robust, tolerating many (random) mutational variations without a reduction in fitness, including mistranslation (560). In general, wide neutral networks correspond to low fitness phenotypes; the highly fit, highly specific antibiotic resistance phenotypes tend to have decreased robustness. When the evolutionary path reaches high fitness peaks, there is a high risk that further changes will produce downhill trajectories.

Neutral variation might also occur because of the effect of phenotypic capacitors, which are proteins involved in cellular networks allowing genetic variation to accumulate in a silent (neutral) state, until the variation is revealed by environmental stress (461, 561, 562). Candidate proteins for effectors of evolutionary capacitance are regulatory genes, networks of chaperones and, in general, proteins with high connectivity with other proteins.

Gene evolutionary trajectories are constrained and sometimes facilitated by the genetic code, which translates genetic information in the protein structure and constrains the mutational exploration of the sequence space (559, 560). Expanded codes might increase the number of antibiotic resistance mutational trajectories (565). In accordance with the Error Minimization Hypothesis, the organization of the pattern of codon assignments is itself the result of natural selection, buffering genomes against the impact of mutations (566, 567). Single base changes in codons can access only about six of the nineteen possible amino acid substitutions. For the beta-lactamase TEM-1, only about 2% of the

possible amino acid combinations in four key positions that increase cefotaxime resistance are in fact accessible (568). However, it has also been proposed that the code has evolved to optimize and ensure adaptive mutations (566, 569). These hypotheses have been tested in the evolution of *bla*$_{\text{TEM-1}}$ beta-lactamase, showing how the genetic code constrains TEM-1 evolutionary trajectories; however, it also restricts mutations with strong negative effects, and therefore orients trajectories toward adaptive benefits (568). Both mutations and indels (insertions and deletions, more frequently insertions) can modify the structure and the molecular fitness of TEM-1 (570). The (without-selection) predictability of the evolutionary trajectory of a given protein is extremely low, however, given a single type of protein always flips between different structural conformations. Thus, the phenotypic consequences of the same mutation or successive mutations in the protein sequence might be unpredictable (571). Conformational dynamics has probably shaped the neofunctionalization and evolution of enzymes (572). Novel techniques mixing experimental evolution and 3D protein structures have confirmed in any case that residue interactions constrain selection of particular sequences (573).

Thus, the number of "functional variant proteins" might be minimal compared with that of all the variant proteins. How large is that minority? Considering only four amino acids are critical for the interaction between two proteins in *E. coli*, only about 1% are functional, suggesting context-dependent mechanisms for certain amino acids, which explains why many variants are not observed in nature (574).

Mutational cost and compensation: mutational robustness. The consequences of mutational events might differ due to the various "levels of phenotypic tolerance" to these genetic changes in a particular organism (genotype). These are levels of mutational robustness (or resilience), affecting the organism's likelihood of maintaining the premutational phenotype. In a sense, a mutation (and the acquisition of a foreign gene)

has the biological meaning of a “change in the cell’s internal environment”, and the maintenance of the phenotypic traits can be viewed as a canalization process.

There are many strategies for mutational robustness, which can include the following: 1) gene redundancy, in which the loss or alteration of function in one copy can be compensated by one of more other copies; 2) domain redundancy, in which only a functional domain of the mutated protein is redundant; 3) gene overexpression, if the mutation has weakened the natural function; 4) presence of genes and pathways with alternative functions; 5) intervention of gene regulatory networks, reducing the influence of the mutated gene in the phenotype, eventually leading to mutated gene silencing; 6) reduction in the need for the mutated gene function by reducing the growth rate; 7) focusing on alternative sources of metabolites or energy by moving to a new environment (plasticity); and 8) the possibility of interactive cooperation with other microorganisms supplying the lost metabolite or function. (318, 575–581). These mutational robustness strategies could be applied to help understand the various pathways involved in the compensatory evolution of the biological costs ultimately imposed by antibiotic resistance mutations.

Gene functional redundancy refers to genes with partially overlapping functions; in other words, degenerated (such as in the genetic code). In the case of allelic forms of the same gene, deciding when a gene evolves sufficiently to “become different” is a difficult task. In a more stringent sense, degeneracy is based on the ability of elements (genes in this case) that are structurally *different* to perform the same function or yield the same output (582). In any case, degeneracy is a main contributor to adaptive flexibility and, in general, to functional robustness and evolvability (583, 584).

Antibiotic resistance mutations: fitness costs. Any deviation in the regular optimality of bacterial fitness in relation to a particular environment has potential

consequences. A mutation in a gene encoding a bacterial function (and antibiotics are designed to act on relevant functions) should have a cost, sometimes cryptic (depending on epistatic interactions, or the environment) or explicit. The mechanisms involved in fitness costs are target-dependent and have been frequently elusive. Mutations resulting in transcription-translation uncoupling and replication-transcription conflicts result in an increased formation of R-loops (three-stranded structures harboring an RNA–DNA hybrid), which cause DNA breaks (585). There are a number of key cellular functions that are hyper-protected by, for example, gene redundancy and mutational robustness, with stronger selection for reduced costs of transcriptional-translational errors (586). The consequences of fitness costs can be expressed as reduced growth, virulence, or transmission (587). Fitness cost effects can also be classified as those influencing growth rate ('trait effects') and those altering genotype frequencies over time ('selective effects') (588). The maintenance over time of a resistance mutation in the absence of antibiotic exposure mostly depends on the environment in which bacteria are located but more specifically on the availability of compensatory mutations, epistatic effects with other genes of the microorganism, including resistance genes (589), or metabolic compensations (149).

Mutational fitness costs are not necessarily proportional to the efficiency of mutations in producing resistance. In fact, fitness costs might decrease with increasing antibiotic resistance (538, 590). The cost of a newly acquired resistance mutation also depends on other mutations in the genome, including other resistance mutations (epigenetic effects) and on the evolutionary history of the organism (591). Most importantly, changes in the conditions for measuring fitness cost (for instance, the use of different culture media) might influence the evolutionary trajectories of resistance mutations (592).

Antibiotic resistance mutations: compensatory evolution. The damage to bacterial fitness ultimately produced by antibiotic resistance mutations can be ameliorated by intragenic or extragenic second-site mutations (593). The more relevant intragenic mutations are those modifying the functional or interactive core of the affected protein, but also "second shell" mutations in neighbor gene domains might have low-level, but significant evolutionary effects (594). Although less explored, gene amplification can also contribute to restoring the fitness of antibiotic-resistant populations (595). Compensatory gene amplification restores fitness after interspecies gene replacements (596). On occasion, these compensatory mutations confer increased resistance, in which case the problem can even be aggravated (597). In other circumstances, however, the mechanisms of fitness cost compensation might offer an opportunity to directly fight antibiotic resistance. The classical view of fitness cost is that it will be reflected in a reduction in the growth rate that will be apparent under any condition. This is likely true when the target and the mechanism of resistance deal with basic elements, such as the ribosome, which is involved in the generation of energy or bacterial biobricks. In these cases, compensatory mutations might be habitat independent (598, 599). However, there are other mutations that can differentially affect bacterial physiology, with those altering bacterial virulence being particularly relevant. In this case, compensatory mutations can be habitat-dependent (580). Fitness costs are relevant for bacterial physiology both when producing an infection and when present outside the patient, as reservoirs that can be sources of infection, which makes it important to determine the causes of compensation in these differing environments.

A final issue concerns the noninherited compensation of the effect of antibiotic resistance. One example of this possibility is the increased expression of a gene that can compensate for the lack of activity in a gene that mutates to acquire resistance. As stated above, this

situation can be the consequence of gene amplification; however, a recent study indicates that this situation can be also be due to overexpression due to changes in regulation (579). Another example is the metabolic shift imposed by the increased expression of efflux pumps, which allows for changes in the respiration rate and the activation of a secondary respiratory chain in *P. aeruginosa:* the nonmutational compensation of efflux pump overexpression by metabolic rearrangement (149).

Mutations competition, cooperation, and founder effects. The emergence of antibiotic resistance is due to selection, which should mean that an already resistant organism is not under selection and does not need to acquire additional mechanisms of resistance. In this situation, if a resistance gene is acquired and spreads quickly in the population, the chances of acquiring a new resistance gene conferring the same phenotype might be low, a situation termed a founder effect, which might explain the low number of different resistance genes acquired by human pathogens compared with the high number of potential resistance genes that can be found in any analyzed microbiome (100, 543). A possible example of this situation is TEM beta-lactamases. TEM-1 was prevalent in *Enterobacteriaceae* until beta-lactamase inhibitors and novel beta-lactams were introduced for therapy. At that moment, when a new selective force appeared, new beta-lactamases were obtained by pathogens, and there was an explosive evolution of TEM variants to cope with this new situation (600). There are, however, other situations in which different genes are established in the population, possibly because different founder effects occur early in different geographic areas (61), as well as sequential events of penetration and extinction of the same gene. The latter situation likely occurs when the donor and recipient are regular members of the same microbiome (601).

A similar situation concerns mutational resistance. The universe of mutations able to produce resistance is several orders of magnitude above those actually selected in

bacterial pathogens. As previously discussed, this can be the consequence of a specifically different mutability (and permissive mutations) of the involved genes (246). However, it can also be the selection strength (602), epistatic influences with other resistance elements, including the consequence of the historical contingency of antibiotic resistance evolution (184), or due to mutant competition. The latter is a specific case of fitness costs. Mutants with higher fitness costs or that are less able to compensate for these costs will disappear more rapidly in the absence of selection than the fitter mutants. In addition, mutants that are fitter in the presence of antibiotics will displace the less fit ones under these conditions; i.e., in treated patients. The latter situation occurs in populations in which the mutation supply is high (i.e., large populations and/or with increased mutation rates). In these populations, several antibiotic resistance mutations might emerge in a relatively short time span and coexist under selection. This leads to the competition between distinct antibiotic resistance mutations, a concept known in classic evolutionary theory as clonal interference (603). Owing to clonal interference, antibiotic resistance variants experience longer fixation times and might be lost from bacterial populations. Clonal interference has been shown to influence the compensation and reversal of antibiotic resistance (604).

Epigenetic epistasis shaping trajectories. Genes encoding for antibiotic resistance are never isolated; there is frequently a network of interactivity with other genes and genetic contexts. Thus, the normal function of a gene cannot necessarily be inferred with certainty from its mutant phenotype. Interactions provide a certain flexibility and malleability to the basic phenotype; such variability increases the chance of obtaining microevolutionary advantages (605). The main genetic context providing flexibility is the rest of the gene sequence; however, other neighbor and eventually co-regulated genes, the genes of the genome of the bacterial host, and probably the genes of other

functionally-linked communities of microorganisms successively influence the expression and consequently the evolutionary trajectories of ARGs. In short, the contribution to an organism's phenotype from one genetic locus might depend upon the status of other loci, and the global genome's flexibility (606, 607).

The study of all these functional gene-gene constellation interactions is the field of epigenetics, referring to heritable (reproducible) changes in gene function that cannot be explained by mutations in DNA sequence, studying the "over-the-gene" events in modifying gene function (608, 609). Variant traits involved in antibiotic resistance will eventually require 'over the gene' interactions, concerted actions of various mutated nonallelic genes to fully express the resistance phenotype. Certainly, antibiotic resistance evolution and evolution in general cannot be explained or predicted without understanding how gene interactions shape adaptive possibilities (182, 490). However, that might be a difficult task; the dependence of the adaptive value of a mutation on the genetic background and the nonadditivity of their functional effects impairs predictability (610). For a given background, phenotypic effects (fitness and resistance level) of resistance mutations can vary substantially depending on the genetic context in which they occur.

The term "epistasis" etymologically means the "act of stopping" (any on-off action) and refers to the phenomenon in which one or more genes influences the function of others. High-order epistasis, when the adaptive value of a mutation is determined by interactions with several other mutations, is a major factor shaping evolutionary trajectories (611). Epistasis for fitness means that the selective effect of a mutation is conditional on the genetic background in which it appears (182, 612, 613). These epistatic interactions might foster or prevent access to evolutionary trajectories toward antibiotic-resistance phenotypes. In experimental evolution assays, for instance, TEM-1 beta-lactamase

2593 frequently evolves to produce cefotaxime resistance by acquiring a few mutations in a
2594 fixed order but not in all repeated replicas of the experiment. Those trajectories starting
2595 with an alternative mutation deviate from the others, tending to be less effective and more
2596 complex (614). In short, differences in directionality can be expressed as *sign epistasis*,
2597 meaning that the sign of the fitness effect of a mutation is under epistatic control; thus,
2598 such a mutation is beneficial in certain genetic backgrounds and deleterious in others
2599 (182). Environmental fluctuation and range expansion (the organism's progeny is
2600 exposed to different environments following population expansion) might increase
2601 epistatic effects and adaptability, accelerating evolution (615, 616). Epistatic differences
2602 in directionality might be contingent, limited to the first stages of the evolutionary
2603 pathways. Impelled by selective forces, random mutations might, in the long term,
2604 converge (adaptive convergence). Based on *in vitro* experiments, it has been proposed
2605 that a single new beneficial mutation might interact with ensembles ("blocks") of other
2606 potential beneficial mutations with positive or negative mutational sign effect, eventually
2607 resulting in the selection of new blocks and the whole evolutionary trajectory (617). A
2608 number of adaptations, including the case of antibiotic resistance, are associated with
2609 epistatic tradeoffs, such that changes in traits that increase fitness in some environments
2610 or situations are deleterious in certain other environments or situations (618). In general,
2611 epistatic events are neutral or negative at early stages of a trajectory and more beneficial
2612 at later stages (610).
2613 Such epistatic interactions not only occur when genes are mutated but could also be due
2614 to variation in gene expression, including among isogenic individuals in a controlled
2615 environment (619). Early mutations in global transcriptional regulators, favored by
2616 environmental changes, might cause extensive changes in the expression of a multiplicity
2617 of genes, which will be subjected not only to positive selection (620) but also to negative

epistatic interactions (621). Stochastic variation in the expression of sets of genes is expected to occur, even in isogenic populations, due to factors that transiently modify the gene function, including DNA methylation, covalent modification of DNA binding proteins, noncoding DNA, and RNA splicing factors. These factors produce epigenetic variation by influencing stochastic fluctuations in cellular components and consequently might have affect he expression of resistance traits.

Epistatic-specific interactions among alleles conferring resistance to antibiotics might reduce or eliminate their expected combined fitness costs, so that some allelic associations result in rapid fitness compensation, which suggests that epistatic fitness compensation might favor the maintenance of multiresistance in antibiotic-free environments (622). These effects are probably more effective in high-order epistasis (in which the effect of a mutation is influenced by two or more other mutations), which facilitates the accessibility of evolutionary trajectories (611). However, other studies have indicated that epistasis remains rare even when up to four chromosomal mutations are combined (623).

Epistasis and hidden genetic variation. Cryptic genetic variation has been considered to act as "evolution's hidden substrate" (624). Gene-gene interactions or epistasis might act without any visible consequences, contributing to the formation of cryptic evolutionary trajectories. Even under conditions of adaptive need (such as antibiotic selection and resistance fitness costs requiring compensatory evolution), the epistatic effect can remain cryptic over many generations, producing evolutionary plateaus (625). Methicillin resistance in *Staphylococcus aureus* has probably evolved cryptically by epistatic effects associated with fitness costs (626).

Mutational Paths in Genes Involved in Antibiotic Resistance

There are a number of mutational paths in genes that already provide antibiotic resistance phenotypes, leading to variant phenotypes, either increasing the ability to resist at higher concentrations of a particular antimicrobial agent, extending the spectrum of inactivation to other antibiotics, reducing the killing (bactericidal) effect of drugs, or reducing the fitness costs of these genes' expression. These paths evolve under the selection imposed by antimicrobial agents and are generally based on mutations in the operative genes or in their promoter sequences. Antibiotics, frequently present in human-influenced microbial structured environments at varying concentrations and with mixtures of drugs in heterogeneous concentration gradients, provoke complex selective landscapes (pharmacodynamic fitness landscapes), which might allow for the possibility of various mutational paths, facilitating pervasive epistasis (627). These paths (or at least those that are able to be detected) appear to be relatively limited in number, and do not necessarily produce the fittest theoretically possible phenotypes (in terms of selectable antibiotic resistance), except for those able to become more abundant in general (628).

We will illustrate the mutational paths of ARGs with three types of examples: 1) mutational paths in target-resistance evolution; 2) mutational paths in inactivating enzyme evolution; and 3) mutational paths (very scarce in this case) in pump-mediated resistance evolution.

Mutational paths in target resistance evolution. Experimental evolution experiments in the presence of antibiotics have demonstrated that mutations in antibiotic targets might follow constant mutational paths, reproducible in parallel lineages. These paths correspond to the "predictable parts" of evolutionary trajectories. Take for instance the case for mutations increasing resistance to ribosome-targeting antibiotics such as tobramycin in *P. aeruginosa*; the patterns of resistance mutations involved might include

common elements (112). A key point in target resistance evolution experiments is the size of the transmission bottleneck, the number of bacteria that are transferred from tube to tube in stepwise passages. Differences in the size of the transfer bottleneck might yield different evolutionary pathways with different final adaptive outcomes; larger sizes likely facilitate the acquisition of a small number of highly efficient target mutations (such as those occurring in the clinical setting), small transmission sizes (including a single cell transfer), and a larger number of less efficient resistance mutations, frequently with higher fitness costs (629). However, survival by these less efficient mutations might favor the acquisition of the more efficient ones. Interestingly, many target alteration mutations demonstrate strain-independent phenotypes across different species (623).

Mutational paths in variant penicillin-binding protein-mediated resistance. The paradigmatic case is beta-lactam resistance in *S. pneumoniae*. Contrary to the primary feeling, directed evolution does not provide significant resistance in most cases. When susceptible bacteria are exposed to increasing concentrations of penicillin, the acquisition of mutations by the penicillin-binding proteins PBP2x and PBP2b, the main resistance determinants, are extremely ineffective in determining clinical antibiotic resistance. Specifically, when the antibiotic target protein is functionally linked in a complex interplay with other proteins (in this case to ensure construction of the cell wall), the maintenance of function requires other cascade changes, which are very difficult to achieve by simple evolutionary events. For instance, changes in PBP2x and PBP2b are only relevant if PBP1 is also altered (630). High resistance to penicillins only occurs if several PBPs (e.g., PBP2x, PBP2a, and PBP1) are altered at the same time. Furthermore, genes such as MurM and MurN involved in the supply of substrate molecules to the PBPs, such as branched muropeptides, should also change to provide “mutated substrates to mutated PBPs” (631). There is a remarkable conservation of PBPs and MurM protein

changes within different *S. pneumoniae*-resistant strains, suggesting that particular PBP-MurM combinations tend to be preserved and might have an independent evolutionary history in particular clones (632).

If sequential acquisition of resistance by mutational changes might be considered a rare event in PBP-mediated penicillin resistance (nearly impossible trajectories), the recruitment of mutations in PBPs and MurM/N proteins leading to penicillin resistance in *S. pneumoniae* occur efficiently by successive recombination events, following horizontal acquisition of chromosomal fragments containing natural or mutant resistant PBPs from neighboring species, such as *S. oralis* (633). The "nearly impossible evolutionary trajectories only" by independent mutation (in the absence of recombination) can be illustrated by the case of the absence of evolution toward penicillin resistance in group A *S. pyogenes*, given this species probably has severe restrictions for genetic interactions (634) involving the CRISPR-Cas9 system, and/or a tightly closed interactive system of communication between PBPs, resulting in new proteins incurring unbearable costs (356, 635). Nevertheless, a recent surveillance study in Canada found two *S. pyogenes* isolates with elevated MICs to beta-lactam antibiotics (636).

There does, however, appear to be a limit to the incremental acquisition of variant or mutant PBPs and other functionally related proteins by transformation and recombination, which steadily increase the levels of penicillin-resistance. Together with the increases in resistance, the biological cost increases with the number of acquired resistant PBP alleles (e.g., in competition experiments with their susceptible ancestor to colonize the respiratory tract) (637).

Another classic case is the evolution of methicillin resistance in MRSA by the acquisition of a gene (*mecA*) encoding an extra penicillin-binding protein (PBP2a) with low affinity to all beta-lactams (638). Acquisition is mediated by the capture of *mecA* by a mobile

staphylococcal cassette chromosome (SCCmec), a resistant PBP that probably originated from *mecA* homologues in *Staphylococcus sciuri*, an ancient group of *Staphylococcus*. In *S. sciuri*, methicillin resistance emerged multiple times (by anthropogenic action?), involving and involved the structural diversification of the nonbinding domain of native PBPs, changes in the promoters of *mecA* homologues, and acquisition of SCCmec (639). The emergence of SCCmec in MRSA was probably associated with exposure to penicillins in the 1940s and not necessarily with exposure to methicillin-oxacillin launched 14 years later (640).

Mutational paths in variant DNA topoisomerases. Following the former case of *S. pneumoniae* examined in the last paragraph, site-specific mutations in a number of target genes (quinolone resistance-determining region mutations) account for incremental resistance to fluoroquinolones (e.g., ciprofloxacin, levofloxacin, and moxifloxacin). *In vitro* serial passage evolution experiments in various organisms indicate that step-wise access to high-level resistance can be achieved with a (relatively) nonrandomly ordered sequential fixation of mutations, following pervasive mutational interactions. In *S. pneumoniae*, it has been proposed that mutations in the ParC subunit of DNA topoisomerase IV (a primary target of fluoroquinolones) should be acquired first, followed by further mutations in the DNA gyrase A subunit, resulting in the formation of a high-resistance phenotype (641). However, both mutated proteins can be acquired in a single recombination event, resulting from horizontal genetic transfer from commensal streptococci, such as *S. oralis* (642). In the presence of double ParC-GyrA mutations, the acquisition of a new mutation in ParE increases the fluoroquinolones' MIC (643). However, this canonical evolutionary path is not universal. Experimental evolution performed in parallel with several lineages derived from a single ancestor pointed to the possibility of different paths. Mutations in the primary target of the selective drug (which

differs with different fluoroquinolones) tends to be selected/fixed first, such as in ParE or ParC in *S. pneumoniae* populations evolved under levofloxacin pressure; however, occasionally other mutations (such as GyrB) can be involved (643). GyrB primary mutations can occur more frequently in other organisms (e.g., *Helicobacter pylori*, *Mycobacterium tuberculosis*); (644, 645). In *E. coli*, the acquisition of a relevant resistant phenotype requires two mutations in GyrA and then single mutations in ParC and/or ParE (629, 646).

As in the case of variant PBPs and beta-lactams, the acquisition of mutations in topoisomerases (eventually altering DNA supercoiling) might influence the strain's fitness and consequently its selectability and potential evolutionary trajectories. By including sequentially different mutations in isogenic *E. coli* strains, a cumulative reduction of fitness was shown with the acquisition of high fluoroquinolone resistance; however, the acquisition of a further mutation (in ParC) might once again increase fitness (i.e., a compensatory mutation), at the expense of reducing the resistance level (597). This result resembles the case of a fitness-compensatory mutation, which in the case of fluoroquinolones acting on *P. aeruginosa*, restores normal levels of DNA supercoiling but involves genes other than those expected to participate in such a function (647).

These fitness effects should influence the outcomes of clonal interference between alleles of mutations influencing fluoroquinolone resistance (643). In *E. coli*, only a limited number of mutational combinations in topoisomerases are found in resistant strains. *In vitro* single-step and multistep selection experiments in parallel replicas of the same *E. coli* strain have indicated a preferential order of selection for particular mutations in GyrA and ParC, whose combinations appear along the selective process. Such an order reflects the higher fitness of those alleles that are selected, as observed in competition experiments (648, 649). The resistance effect of topoisomerase mutations can be enhanced by further

mutations in ParC or ParE or in efflux pumps. The order of mutations obtained under serial passages faithfully correspond to that detected in clinical strains, particularly using large transmission bottlenecks (629, 648). We can postulate the coexistence, under *in vivo* situations, of different "transmission bottlenecks" and different selective antibiotic concentrations, such that low-level resistance mutations might facilitate the acquisition of efficient target mutations.

Mutational paths and target gene conversion. As presented in the previous paragraphs, the acquisition of a target gene mutation might influence, at least in some cases, the evolutionary paths of neighboring strains by horizontal gene transfer and recombination. In the case of homologous repeated genetic sequences of a target gene in a single cell, the mutation acquired in a copy (generally producing low-level resistance) might easily be reproduced by intragenomic recombination in the other copies (providing a high-resistance phenotype). This phenomenon is known as "gene conversion", assuring non-reciprocal, ensuring the nonreciprocal transfer of information between homologous sequences inside the same genome. For instance, single-mutated rRNAs easily produce antibiotic resistance to aminoglycosides when the other copies of rRNA sequences remain unchanged; the resistance mutation spread by gene conversion (650). In the case of linezolid (oxazolidinones), the G2576T resistance mutation in domain V of 23S rRNA occurring in a single copy (very low-level resistance) propagates in the other copies by RecA-dependent gene conversion, facilitating access to high-level resistance (651).

The influence of gene conversion in the evolution of nontarget genes (for instance, providing antibiotic detoxification mechanisms) has been less explored, but there are a number of cases in which a resistance gene (e.g., beta-lactamase) evolves by gene duplication, or the same gene is present in different or multicopy plasmids. In these cases,

the possibility of gene conversion in the intracellular propagation of advantageous mutations occurring in a single gene is an interesting possibility.

Gene conversion might also contribute to restoration (repair) by recombination of the wild sequence of the susceptible phenotype and, in general, to the concerted evolution of multigene families (652), which would be an easy method for reverting resistance and compensating its costs.

Mutational paths in evolution of detoxifying enzymes. Directed evolution coupled with structural analysis can be employed to predict future mutations that lead to increased antibiotic resistance. The impact of mutations is context-dependent and reflects a complex network of interactions between multiple residues within a protein, which is certainly the case in beta-lactamases. In fact, different "modular communities of associated mutations", visible in networks, appear to occur for broad-spectrum, extended-spectrum, and inhibitor-resistant beta-lactamases (653).

Weinreich et al. focused on the evolutionary possibilities of TEM beta-lactamase in *E. coli* (654) employing a model that included five-point mutations in the basic TEM-1 allele, which is able to move the resistance phenotype from aminopenicillin-only to high cefotaxime resistance. Evolution to cefotaxime resistance might follow any of the 120 theoretical mutational trajectories linking these alleles. It has been demonstrated that most of these trajectories (85%, 102 trajectories) are inaccessible to Darwinian selection and that many of the remaining trajectories have a negligible likelihood of being traversed, such as contained fitness reduction and neutral steps, including sign-epistatic interactions resulting in significantly reduced chances of being followed by natural selection (653, 655).

The effect of sign epistasis on adaptive trajectories, particularly antagonistic pleiotropy (when the mutation providing resistance to antibiotic A increases susceptibility for antibiotic B), is particularly critical when the bacterial organism is subjected to fluctuating selective environments, which occurs frequently in hospitals (the same epidemic or endemic clone moves from patients treated with drug A to those treated with drug B) or in sequential therapy with the same patient, including de-escalation strategies. To overcome antagonistic pleiotropy, new ("modulatory") mutations are required (480).

Accessible (possible) trajectories are not based only on advances in the resistance level or on the spectrum of antibiotic inactivation. The variant protein should not only be active but also sufficiently stable, and a number of apparently neutral mutations, including suppressor mutations, are required for reorganizing the topology once "advantageous mutations" have been achieved (i.e., stabilizing mutations) (656). The accessible "protein space" depends on the conservation of a relatively low number of possible protein folds (fewer than 10,000?), which depends on the amino acid sequence (90, 657). The variant protein might evolve to a successful protein by improving its localization in the cell. It has been suggested that the success of the metallo-beta-lactamase NDM-1 is due to its lipidated structure, facilitating anchoring to the bacterial membrane (658, 659).

Inactivating enzymes: the case of beta-lactamases. Evolutionary biology often assumes that, for any protein, natural selection has already explored all adaptive options for achieving optimal efficiency, and any protein variant would be counterselected provided the environmental conditions remain stable (purifying selection). However, if the environmental conditions suddenly change, the protein activity might not be as efficient (bottleneck), and consequently a series of variant proteins could be selected until they once again achieve the optimal fitness peak (positive selection). An excellent model of the so-called perfect enzyme is TEM-1 beta-lactamase.

Stiffler et al. (660) mutagenized all positions in TEM-1 and found no change that increased the MIC of ampicillin, although 2% of these changes increased the activity of cefotaxime. Nevertheless, easy-to-implement, deep-sequencing technology and metagenomic studies from human (52, 661) and nonhuman (44, 662) sources provide increased evidence of a rapid increase in new variants into known beta-lactamase families. Over the last decades, there has been an explosive growth in the description of new beta-lactamases (663) and variants of these new enzymes, suggesting continuous changes in selective pressures.

Although the diversity of TEM enzymes is high (currently 225 variants), affecting up to 32% of amino acid positions, several authors have demonstrated that only 13–16% of the positions in TEM-1 beta-lactamase do not tolerate substitutions (the enzyme's core), with critically or drastically reduced hydrolytic activity. More diversity should therefore be present in the real world, which is not the case. The reason for this difference is that many changes have a neutral effect (664), i.e. they do not offer phenotypic advantages but do not therefore lose activity. These changes could therefore only be amplified by stochastic events (drift) in small bacterial populations. It also has been shown that the neutrality of these changes is itself conditional on the selection strength; i.e., under weak selection (for instance, low ampicillin concentrations), the vast majority of mutations are statistically neutral; under strong selection (high ampicillin concentrations), however, the enzyme's overall fitness cost and the proportion of variant alleles is dramatically increased (660). Cefotaxime resistance mutations can be found among ampicillin-neutral mutations selected under low ampicillin exposure and rarely among those selected with high concentrations, which might be explained by the decrease in robustness of these latter variants. Deng et al. observed that the impact of mutations is highly dependent on the

enzyme global stability and accessibility of residues, with buried positions being less tolerant of substitution than surface positions (665).

The concepts of strong selective pressure, fitness, and global protein stability are closely related (666). For instance, it well known that mutations influencing the beta-lactamase omega loop, which are found in oxyimino-cephalosporin-resistant variants, reduce enzymatic stability in TEM (667) and CTX-M beta-lactamases (668). The loss of stability caused by the selection of R164H/S/C in TEM variants or P167S/T in CTX-M is eventually compensated by other mutations reducing the instability caused by the main mutation, ensuring the persistence of the new selected variant. In TEM variants carrying the R164S change (e.g., TEM-12), the introduction of the M182T (TEM-63) secondary mutation was beneficial, stabilizing the enzyme, increasing its half-time, and consequently increasing the ceftazidime resistance (665). If this M182T mutation is introduced into TEM-1, however, there is no increase in beta-lactam resistance (0.08 mcg/ml in wild-type TEM-1 to 0.06 mcg/ml in TEM-135), because the wild-type enzyme is already very stable (669, 670) thereby providing a good example of the role of contingency in the evolution of antibiotic resistance. These compensatory substitutions will therefore only be selected depending on the genetic background (sign epistasis) (669). Similar findings were observed with the A77V mutation in CTX-M-3/CTX-M-1or CTX-M-14 and their evolved variants (181, 557). In evolution experiments in serial passages with CTX-M-3, the A77V was detected after the P167S mutation was fixed in the population (671). These compensations influencing the enzyme's stability might allow the buildup of strong dependencies among mutations.

Based on the assumption that the presence of two mutations in the same sequence could be a marker of a potential functional interaction, Guthrie et al. performed a computational prediction using a network among all mutations identified in TEM variants (653) and

found a complex framework with many interactions. However, only a few interactions were strongly connected (positive epistasis). These associations between mutations were considered as signs of evolutionary adaptation pathways.

Weinreich et al. conducted early studies to understand the impact of adaptive pathways in beta-lactamase evolution and demonstrated that TEM-1 beta-lactamase evolution towards a super-effective cefotaxime-hydrolyzing mutant (carrying five mutations with respect to the wild type TEM-1) was only possible across 18/120 (15%) mutational pathways, revealing that there is a predetermined fixed order in the incorporation of each mutation (654). This situation occurs particularly during the first three mutations, a consistent finding in repeated experiments (614). Certain other trajectories are the result of an epistatic clash between mutations. An initially deleterious mutation might be the key for achieving a more effective (high cefotaxime MIC) allele, a mutation that is a gateway for reaching an otherwise relatively inaccessible area of sequence space, where more efficacious enzymes can be found (503).

The improvement in MIC provided by the enzyme is not the only evolutionary goal for antibiotic-inactivating proteins such as TEM enzymes. Protein stability is also an important driver (555); highly stabilized variants of TEM-1 beta-lactamase exhibit selective rigidification of the enzyme's scaffold while the active site loops maintain their conformational plasticity (672). These findings support the view that, although many hypothetical evolutionary possibilities could be suspected, only a small number of them are feasible according to Darwinian natural selection. Moreover, these results agree with the evolutionary impact of compensatory mutations (also called global suppressors, such as M182T and A77V), which could never be selected as the first change.

Novais et al. (181) studied the fitness landscape in CTX-M, identified those positions under positive selection, and constructed all mutational combinations. Similar to the

Weinreich group's conclusions, only a few trajectories were necessary from CTX-M-3 until a more efficient enzyme for hydrolyzing ceftazidime was reached (CTX-M-58). Nevertheless, the authors observed that the number of evolutionary trajectories could be increased if the environment fluctuated between two antibiotics, such as ceftazidime and cefotaxime. Other authors have also recently suggested that enzymes with high activity would be evolutionarily favored under fluctuations in the distribution of their beta-lactam substrates (660). This concept underlines our proposal that antibiotics are both selectors and accelerators of variant diversity (673). Considerations of the impact of fluctuating environments, including two or more antibiotics, and the differences imposed by variable concentrations exemplifies our limited capacity for predicting evolutionary trajectories of antibiotic resistance (485). However, future tools can be envisaged that mimic fluctuating fitness landscapes to help determine why particular paths are taken in particular environmental conditions (502).

In the previously mentioned study by Guthrie et al. (653), the authors also found clear evolutionary segregation in various mutational subnetworks, corresponding to three distinct phenotypic categories in TEM-1: broad-spectrum, extended-spectrum, and beta-lactamase inhibitor resistance, suggesting an antagonistic pleiotropy between different resistance phenotypes. This phenomenon was also observed by our group, using ROB-1 from *Haemophilus influenzae* and CTX-M (472, 473), a finding that serves as an introduction to the topic of evolutionary constraints, which could be related to the antagonism observed between different resistance phenotypes (the selection of mutations involved in the resistance to beta-lactam plus beta-lactamase inhibitor combination yields an enzyme more susceptible to oximino-cephalosporins) and the antagonism between two mutations involved in the same resistant phenotype. For instance, the mutations P167S/T and D240G in CTX-M, which are involved in the phenotype of ceftazidime resistance in

CTX-M (CTX-M-58 and CTX-M-32 variants, evolved from CTX-M-1 or CTX-M-42 and CTX-M-15 evolved from CTX-M-3), are mutually exclusive (181). Similarly, the G238S and R164S mutations in TEM variants selected under antibiotic pressure with oximino-cephalosporins show a case of negative reciprocal sign epistasis (674). This mutational antagonism reveals alternative evolutionary solutions in response to the same selective pressures (antibiotic pressure with oximino-cephalosporins), suggesting that the fitness landscape contains more than a single adaptive peak, probably including several evolutionary paths. The study by Salverda et al. employed twelve experimental evolution assays using TEM-1 and confirmed that the AG238S mutation was more frequently associated with E104K as a secondary mutation, whereas when the first mutation was R164S, the second mutations were frequently E240K and A237T (614), suggesting two separate and incompatible trajectories. This observation also occurs in natural environments (653).

The initial random substitution of one of those mutations therefore suggests that only a small fraction of all adaptive trajectories could be selected. Similarly, the study by Novais et al. that analyzed the two main mutations (P167S/T and D240G) involved in ceftazidime resistance in CTX-M observed the mutational antagonism between them, which also represents two separate trajectories. Moreover, the authors suggested a third path of ceftazidime resistance, excluding P167S/T and D240G but including other mutations under positive selection and conferring low-level resistance. This third path is represented by the trajectory from CTX-M-3 to CTM-M-1, increasing the MIC of ceftazidime four-fold (181). This alternative pathway could have more epistatic interactions with the two antagonist trajectories.

The possibility of two or more separate outcome trajectories in response to a common selective pressure might be the consequence of privileged connectivity (the R164 position

has seven interactions with neighboring residues, whereas G238 has only two) (674). The high connectivity of R164 induces an easier collapse of this interaction network when this position is mutated. In contrast, G238 shows a ten-fold faster evolutionary rate than the R164 position (675). This observation is confirmed in natural conditions and experimental evolution assays (614). In the case of P167-D240 positions in CTX-M, the D240 trajectory tolerates numerous changes, smoothly increasing the MIC of ceftazidime. In contrast, P167S/T dramatically increases the MIC of ceftazidime, but practically all successive changes yield a loss of optimal fitness peaks, explaining why a higher proportion of mutants selected in nature are those that carry the D240G mutation.

If the initial mutation determines the evolutionary trajectory, are there factors that affect the choice and selection of one or another trajectory or that depend only on random events? The fastest fitness landscape depends on the relative magnitude of the mutation rate and population size (676, 677). In small populations and low mutation rate situations, the best choice is the shorter trajectories to reach the fitness peak (such as P167S/T mutation in CTX-M), the so-called "survival of the fittest" as the most paradigmatic view of Darwinian evolution. In contrast, in large populations and high mutation rates, the most successful strategy is large evolutionary trajectories in time (such as the D240G mutation in CTX-M), the so-called "survival of the flattest" (678), because in these conditions the fittest organisms are those showing the greatest robustness against the deleterious mutations (679). According to clinical evidence, the survival of the flattest in antibiotic resistance is generally the most successful strategy, because the antibiotic bottlenecks select microorganisms with high mutation rates (273).

The evolution of *K. pneumoniae* carbapenemases (KPCs) have also been observed to lead to new variants of KPC-2 or KPC-3 that reduce carbapenem MICs but also affect the inhibitor capacity of avibactam (680, 681). This finding has been associated with the

presence of this carbapenemase in the high risk-clone ST307 of *K. pneumoniae.* The antimicrobial drug pushing the evolution of beta-lactamases might not coincide with the one that has emerged subsequently with the use of a new antimicrobial. For instance, it could be expected that the selection and evolution of VIM-type carbapenemases could correspond to the increased use of carbapenems, but surprisingly ceftazidime, an older antibiotic, is responsible for this process (193). Dissemination and evolution of beta-lactamases strongly depends on their adaptability to the organism harboring the enzyme, given that the signal peptide sequence expression dictates the consequences on bacterial fitness of each particular host (659).

The case of aminoglycoside-inactivating enzymes. Aminoglycoside resistance by inactivating adenyltransferase (AAD), phosphotransferase (APH), and acetyltransferase (AAC) enzymes provides another example of available evolutionary trajectories. Most of these enzymes (as has been shown in APHs) probably derive from Actinomycetes ancestors, and horizontal transfer by capture in integrons, transposition, and conjugation has possibly contributed to allelic diversification (682–684). In contrast to the case of beta-lactamases in which mutational evolution in the first detected classic enzymes (e.g., TEM, SHV, OXA, VIM, CTX-M) has contributed to expanding the spectrum of inactivated compounds, no such contribution has apparently occurred under aminoglycoside clinical exposure. Hypothetically, several of these enzymes could have ameliorated their abilities to inactivate other aminoglycosides; however, this phenomenon is not comparable. The *in vitro* evolution of APHs acting on old aminoglycosides (kanamycin) has indeed produced variants with increased inactivation potency toward newly introduced aminoglycosides such as amikacin and isepamycin (685). It has been proposed that these strains do not evolve in the clinical setting, either because they produce high fitness costs or because they compete with many other amikacin-

inactivating enzymes already present in natural populations, including clinical strains. The more frequent ones include the AAC(6') enzymes, which probably have emerged independently; at least three families are detectable through phylogenetic analysis. The potential of the *aac(6')-Iaa* gene to increase resistance to tobramycin, kanamycin, or amikacin and to acquire resistance to gentamicin was assessed by *in vitro* evolution experiments, which did not succeed in obtaining alleles with increased resistance (686).

Mutational paths in efflux pumps. Mutations in genes encoding resistance determinants can increase the phenotype of resistance, which, in the case of antibiotic-inactivating enzymes, occurs mainly by increasing the affinity of the enzyme to its antibiotic target. Nevertheless, the same affect can be achieved by just increasing the amount of the resistance determinant. Increased TEM-1 production has been described as the first cause of resistance to the combination amoxycillin/clavulanate (284), and the increased production of chromosomally encoded beta-lactamases due to mutations in their regulators is a frequent cause of resistance to beta-lactams (322, 687).

A similar situation appears to apply for chromosomally encoded MDR efflux pumps, which are expressed at low levels under regular growing conditions; however, high-level expression can be achieved through mutation in their regulatory elements. Efflux pump overexpression has actually been observed in experimental evolution conditions (59). The interplay between intrinsic and acquired resistance to quinolones has been shown in *Stenotrophomonas maltophilia* and in other clinical resistant isolates evolving under antibiotic treatment (688). The increase of efflux-mediated resistance in *P. aeruginosa* during antibiotic treatment occurs in patients experiencing nosocomial pneumonia. Unlike other resistance determinants, MDR efflux pumps are nonspecific; each independent efflux pump can extrude a variety of antimicrobial compounds belonging to different structural families. Under this situation, improving the affinity for one

compound might reduce the affinity for other substrates. In other words, increasing resistance to certain drugs might decrease resistance to others, a situation described in the case of AcrB. The study of the genomes of pretherapy and posttherapy MDR clinical isolates *of Salmonella* Typhimurium showed that a mutation increasing AcrB activity for extruding quinolones had been selected posttherapy (689). AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity. This mutation made *Salmonella* hypersusceptible to other antimicrobials, resulting in the mutation being unlikely to be selected under combination or sequential therapy. A number of examples have recently been published showing that antibiotic resistance can be acquired by modifying the efflux pump structure (690). However, nearly all studies on resistance and MDR efflux pumps have focused on the overexpression of these resistance determinants, which increased resistance to every toxic compound extruded. Whether mutations that improve their activity are equally relevant remains to be established (691).

Evolutionary trajectories of gene complexes involved in antibiotic resistance.

A number of antibiotic resistance phenotypes do not depend on the presence of particular ARGs and their variants but integrate a functional complex array of several genes (complex traits). Complex genetic ensembles might arise by modularity, whereas certain genes tend to be genetically and functionally organized into groups. It has been suggested that such constructions are dependent on directional selection and improbably by drift or stabilizing selection (692). The expression “complex traits syndrome” refers to nonclustered genetic associations involving genes in different locations of the genome, whereas operon genes are co-transcribed under the control of a single promoter to a polycistronic mRNA molecule. A typical case is an operon of functionally linked, co-regulated genes, such as in VanA-type vancomycin-resistance and mercury-resistance Mer operons (693). The buildup and instability of operons, i.e., the “life-cycle of operons”

(694), is a complex issue (695). Operons probably evolve from several ancestral intermediary states that have certain functionality, which are improved in function and regulation in later stages by the acquisition of new genes (696).

In many cases, several horizontally transferred genes might be acquired simultaneously. This complex transfer occurs more frequently for functionally interdependent genes, probably because spatial and functional clustering ensures the expression of a function requiring different genes (697). The horizontal transfer of complete operons is not an infrequent event, consistent with the "selfish operon" hypothesis (698). Resistance operons are frequently inserted into mobile genetic elements. Operon promiscuity might have contributed to the evolution of these complex traits, favoring the acquisition of foreign ortholog genes (even from taxonomically diverse organisms), which might *in situ* displace less fit ancient genes inside the operon (699). On other occasions, resistance operons might have evolved via independent assembly, in part from horizontally acquired genes. An integron-like origin of resistance operons can also be suggested (700). Integrons includes a site-specific (*attC*) recombination system capable of integrating and expressing individual genes contained in mobile gene cassettes, leading to gene strings. Successive acquisition and local shuffling of genes of different origins might have produced operon-like structures, fixed through the subsequent loss of *attC* sites and then mobilized outside of the integron array and selected in particular organisms after antimicrobial exposure.

Due to the need for an integrated function and according to the "complexity hypothesis" (701, 702), horizontal gene transfer is less frequent in informational genes (such as those that co-evolved as determining complex processes such as transcription and translation and are typically interconnected members of large, complex systems) than in operational genes (which are more involved in housekeeping functions). The difficulty in acquiring

informational genes also depends on the orthogonality dynamics. The building-up of complex functional multigene sequences in antibiotic resistance mirrors the general assembly patterns of genomic functional regions. Such an organization should have a chronological structure, resulting from a sequential, directional gain of function. According to a number of authors, predicting these gains after a network modeling analysis should be possible (703).

Costs and Benefits of the Acquisition of Foreign ARGs and Functions: the Question of Orthogonality

Any acquisition of foreign genetic material represents a danger to the functional integrity (and identity?) of the bacterial cell. Such integrity tends to naturally be preserved, and the compartmentalized life of organisms requires robustness to tolerate genetic invasions that frequently create fitness costs. However, these invasions provide evolutionary novelty beyond the adaptive possibilities of the isolated organism.

The issue of orthogonality is worth discussing here, a term borrowed from vector theory in mathematics and widely employed in synthetic biology and computational sciences in systems theory. Orthogonality implies a factual independence between otherwise coexisting systems (704). To be functionally active and not impose fitness costs, a resistance gene (function) should not interfere (should be orthogonal) with the ensemble of genes (functions) of the receptor organism. Full orthogonality is however unrealistic, given that the incoming gene necessarily competes with the cell's replication and translation machinery, and the resistance function should be expressed in interaction with the cellular structures. There is a paradox to be considered here: are resistance genes from distant organisms better tolerated than resistance genes from closer lineages?

Codon usage compatibility between foreign genes and recipient genomes is an important prerequisite for assessing the selective advantage of imported functions and the associated fitness and therefore to increase the likelihood of fixing genes acquired via horizontal gene transfer events (705). However, this cost can be minimized both by in *cis* changes in the acquired gene promoter or in *trans* changes in the host genome, without introducing mutational changes in the antibiotic resistance gene (706). Ribosomal mutations might allow the efficient expression of exogenous genes that are nonoptimal for the tRNA repertoire of the new host (707). There are many decontextualized resistance genes (14). It has been reported that directional selection on a highly constrained gene previously under strong stabilizing selection was more efficient when it was embedded within a network of partners under relaxed stabilizing selection pressure (708).

The ensemble of the genes in a genome (from core genome to pangenome) constitutes something like an integrated ecosystem, the functions of each gene contributing to the formation of an "environment" where the functions of all others should be accurately incorporated in a common, robust ensemble. Gene variation, or foreign gene acquisition required for survival in the case of antibiotic resistance is always a stress situation forcing to reshape evolutionary trajectories to minimize risks of extinction.

EVOLUTIONARY TRAJECTORIES OF MOBILE GENETIC ELEMENTS HARBORING RESISTANCE GENES

MGEs of prokaryotes can be defined as any type of DNA coding for proteins that mediate the movement of DNA either within the cell genome (intracellular mobility) or between bacterial cells (intercellular mobility). Most MGEs have been classically categorized in terms of their basic genetic content, mechanistic transfer properties, or regulatory aspects;

however, the categorization of MGEs is difficult ontologically (and thus taxonomically), because the frequent modular exchange of fragments between elements often results in mosaic entities or genetic configurations with distinct functional properties (709–713). The total pool of MGEs, either in cells, populations, species, or multispecies genetic exchange communities, constitutes the mobilome (714). The ecological context appears to determine the abundance and diversity of mobilomes as reflected by MGE enrichment in the gut, oral microbiomes and particular taxa (712, 715). Such robustness indicates that contemporary MGEs/mobilomes were not born with antibiotic resistance but that their current abundance, diversity, and complexity is the result of a cumulative series of anthropogenic interventions, a "history of significant events" that continuously shape the evolutionary paths and trajectories of AMR.

In this section, we will focus on the ecology and evolvability of MGEs, which have a major impact on the evolution of AMR; namely, plasmids, transposable elements, integrative-conjugative elements (ICEs), and bacteriophages. We will also highlight the blurred borders between some of these categories (713, 716) and the mechanisms that maintain robustness in the context of AMR. Remarkable gene recruitment systems such as integrons have been revised elsewhere (717). and are analyzed in the context of the MGEs in which they are usually embedded. We will briefly address the interesting case of mobile promoters, MGEs transferring entirely noncoding DNA sequences, resulting in horizontal regulatory transfer (718), which can increase ARG expression.

Ecology and Evolution of Mobile Genetic Elements

Plasmids. The term "plasmid" was first introduced by Joshua Lederberg in 1952 to define any extrachromosomal hereditary determinant (719). The demonstration of transferability of antibiotic resistance phenotypes (alone or in combination) in isolates from epidemics caused by multiresistant *Shigella flexneri* in Japan in the 1950s (443),

from *Salmonella* in English farms, and from *Staphylococcus aureus* in European and Australian hospitals in the 1960s led to the landmark discoveries of non-Mendelian infective heredity (720, 721), the players involved in this process (initially episomes, resistance plasmids, R plasmids, and R factors) and the later identification of transposable elements. In addition to the self-transferability and the ability to accumulate ARGs, these early studies also highlighted the plasmids' ability to cross species barriers, generate novel entities resulting from recombination events, and increase the copy number (and thus, the mutation rate) after gene acquisition, making them unique among all the MGEs described to date (443, 722). The biology and epidemiology of plasmids have been extensively (and increasingly) analyzed since their first description (723–729). However, the role of plasmids in the robustness and evolvability of bacterial populations has been poorly addressed due to the limitations of technical approaches to fully characterize plasmid sequences.

Plasmid categorization is based on the diversity of replication (729–732) and conjugation machineries (33, 729, 733, 734), enabling the application of a common nomenclature that can help track ARG propagation and analyze the epidemiological and biological features of various families over decades. A recent comprehensive phylogenomic analysis based on pairwise identity of the 10,000 plasmids available in public databases demonstrates how plasmids cluster in coherent genomic groups called plasmid taxonomic units (PTUs), which are similar in concept to bacterial species by the analogy of PTUs with bacterial operational taxonomic units (716). This approach provides a more robust plasmid classification (PTUs are poorly correlated with "classical" incompatibility or mobility families), revealing a gradient of host ranges for different PTUs (not all plasmids are equally involved in HGT and therefore have a differing effect on the propagation of adaptive features). This issue has been widely analyzed but poorly addressed in the

literature because the host range has been based on very few plasmid representatives (735).

More than half of the PTUs defined by Redondo-Salvo et al. (716) are associated with Enterobacterales, Bacillales and Lactobacillales, which reflects the predominance of plasmids in the gut and oral microbiomes of humans and animals (736, 737), and are thus involved in AR. Plasmid diversity has been comprehensively analyzed in various taxonomical groups, including Enterobacterales, *Acinetobacter* (738, 739), *Pseudomonas*, *Staphylococcus* and *Enterococcus* (33, 729, 740), *Neisseriaceae* (741), and *Vibrionaceae* (742); however, the Enterobacterales are by far the most analyzed plasmid entities. A gradient of host ranges for different PTUs has been inferred from comprehensive genome databases, with the number of mobilizable and conjugative plasmids able to propagate between species of different bacterial genera and families being higher than that of plasmids able to move between orders (e.g., 9 PTUs that include emblematic IncL/M, IncN1, IncW, IncHI2, IncX1), classes (e.g., PTUs-IncC, previously known as A/C; and PTU-Q2) and phylum (e.g., PTU-P1). Epidemiological data complement (and confirm) the heterogeneity of plasmidomes in bacterial populations, from species to the microbiome level (33, 65, 743, 744), which is influenced not only by the plasmids' "conduciveness" but also by that of the host (745). Maintenance of plasmid heterogeneity has obvious benefits for the robustness and evolvability of bacterial communities (746). Such plasmid heterogeneity enables a rapid response to antibiotic challenges in connected environments through broad host plasmids that trigger ARG propagation between host-adapted bacterial populations (747).

In principle, plasmids impose a fitness cost on the cells where they are located. This fitness cost derives from the cellular maintaining, transcribing, and translating of plasmid genes, from the interference between chromosomal and plasmid regulators and due to the

fitness-lowering effects of plasmid-encoded proteins (748, 749). This fitness cost is critical for explaining plasmid evolvability. The generation and maintenance of adaptive plasmid variants has been explained by compensatory evolution to ameliorate plasmid cost (750), which involves chromosomal or plasmid mutations, the transport of partitioning genes or toxin–antitoxin systems genes that directly enhance plasmid stability (751), enhanced infectivity, epistasis between plasmids that often co-infect the bacterial cell (752), and source-sink dynamics in multispecies populations (753). Mutations leading to a reduction in plasmid fitness costs tend to be based on the chromosome if vertical transmission of the plasmid predominates over horizontal transmission. Thus, infectious transmission and compensatory evolution might be competing evolutionary trajectories (754).

One remarkable feature of plasmids is that they typically are kept, on average, at more than one copy per bacterial chromosome, which is particularly true for small, multicopy plasmids that have been shown to accelerate the evolution of antibiotic resistance by increasing the rate at which beneficial mutations are acquired (303). When new mutations appear in multicopy plasmids, the mutations coexist with their ancestral allele during a number of generations that are proportional to the plasmid copy number. This coexistence allows plasmids to provide simultaneous resistance to different antibiotics of the same family, overcoming the restraints imposed by tradeoffs in the evolution of antimicrobial resistance genes (258). These features highlight multicopy plasmids as important catalysts of bacterial evolution. The widespread ColE-1-type and IncQ plasmids are the paradigm of multicopy plasmids associated with the acquisition and spread of ARGs in *Enterobacterales*, *Pasteurella*, *Vibrio*, and *Aeromonas* (755, 756). An increase in the copy number of conjugative plasmids can occur in the presence of antibiotics to enable

gene-dosing effects and to facilitate the acquisition of a costly phenotype in heterologous hosts (757).

Comparative genomics of available plasmids help infer subsets of variants that would be adaptive for evolutionary lineages, given that certain changes cannot be recurrent or infrequent for the evolutionary lineage and thus are unable to persist in the long term (758). Early plasmids of *Enterobacteriaceae, Pseudomonas*, and *Staphylococcus aureus* encoded resistance to the heavy metals mercury, cadmium, and arsenic between the 1900s and 1930s and to the antibiotics sulfonamides, tetracyclines, penicillins, and streptomycin, widely employed since the mid-1940s, suggesting the ARG acquisition in a few preexisting antimicrobial-resistant plasmids (759–763). However, the evolutionary trajectories vary among different plasmid categories and plasmidomes, ranging from highly conserved backbones, such as plasmids W, C (formerly A/C) and P1 (764–766), to highly variable subtypes within classical F, I, and X families (767–770), which could be distinct PTUs. (716).

Plasmid gene networking is a major evolutionary feature of resistance plasmids. ARGs located in plasmids are embedded in other MGEs inserted in the variable region of the plasmid genome, often clustered in multiresistance regions (771). ARGs are often located on various plasmids that frequently coinfect bacterial populations (772, 773). A dense network of extensive plasmid exchange involving genes, MGEs, or chromosomal regions facilitates the adaptation and evolvability of both plasmids and bacterial host populations. As a first possibility, identical genes/MGEs can be captured by various PTUs available in the ecosystem, which can occur by recombination between plasmids or by independent acquisitions from common or different sources. Plasmid and host "conduciveness" (favorable interactions) varies between populations and highly influences the propagation of different ARGs. Second, plasmids can recombine, yielding multiple replicons that

enable plasmids to replicate in different hosts. Multireplicons are frequently involved in the propagation of ARGs, such as the F plasmids in *E. coli,* the nonmobilizable plasmids of *Neisseria gonorrhoeae* (741), the Inc18 chimeras, and the pheromone-responsive plasmids and RepAN plasmids in enterococci (33, 729). Third, plasmids can mobilize chromosomal regions or elements carrying ARGs and/or virulence factors *in trans.* Emblematic examples include IncC plasmids (previously A/C) of Proteobacteria, associated with the transfer of *Salmonella* and *Proteus* genomic islands (SGI1PGI1 elements) and other *Vibrio* MDR-GIs to *Salmonella*, *Proteae*, *Vibrio*, *and Shewanella* (774–777); F plasmids of *E. coli* with high-pathogenicity islands (778); and Inc18 plasmids of *E. faecalis*, associated with the transfer of large chromosomal regions (779).

Transposable elements. Transposable elements (TEs) are tightly regulated and conditionally expressed mutagenic elements whose main physiological and evolutionary significance is to link nonhomologous DNA (780), which occurs through the flanking of a nonhomologous sequence by mediating the cointegration of two replicons (which can result in composite platforms) or by mediating arrangements (insertions, deletions, inversions, or translocations) through HGT or recombination. TEs are frequently found in plasmids, ICEs, bacteriophages, and chromosomes and can transfer between hosts by moving from chromosomal sites to mobile DNA molecules (MGEs) and *vice versa*, thereby influencing the trajectories of antibiotic/xenobiotic resistance and the evolvability of clonal lineages and MGEs. TE activity constitutes one of the more important forces that affect the evolutionary trajectories of antibiotic/xenobiotic resistance in human and animal pathogens, as well as the trajectories of other MGEs and bacteria.

Despite the ubiquity and diversity of TEs (781), the number of different chemical mechanisms employed in TE movement is surprisingly limited, with many divergent TEs sharing a similar mechanism. Nonrandom distribution is a common attribute of TE

insertions; however, target site preference for insertion site and transposon immunity vary among TEs, which, in addition to natural selection, determines the distribution of various TE entities and thus their dissemination highways and occurrences of ARGs and other adaptive traits. TE self-regulation modulates the extent of damage in the host, with low activity under normal circumstances and activation under stress, which could ensure survival in offspring. However, the TE content can vary with the TE element, given that transposition immunity (Tn*3* and Tn*7*) plays a relevant role in these entities' survival and dissemination.

Many TEs were initially discovered due to the carriage of ARGs (782, 783). Categorization of transposable elements has been based on differing criteria, mainly the diversity of the transposases (Tpases) and the ability to self-mobilize (784). However, borders between TEs are unclear, and there have been an increasing number of reported elements involved in AMR that do not fit into traditional classifications (712, 713, 785). This section reviews the heterogeneity of the elements (diversity) and the adaptive strategies for ARG evolvability, highlighting the interactions between elements. Although the diversity of TE effects is widely documented, the relevance of interactions with the host is largely unknown, and different relationships, from mutualism to parasitism and co-option, have been suggested.

Insertion sequences and insertion sequence derivatives. Insertion sequences (ISs) are the simplest autonomous MGE in bacteria, comprising only one or two proteins needed for their own transposition. In addition to the classical IS model, this category currently includes a variety of IS-related TEs that share various levels of similarity with ISs, all widely distributed and associated with AMR. ISs are categorized in well-defined major families associated with different transposase types (e.g., DDE, DEED, HUH, Ser Tpases). Nonclassical ISs or "IS-related TEs" comprise self-

transferable and nontransferable elements. The self-transferable group includes i) ISs with accessory genes regulating the transposition (e.g., IS*21*, IS*91*, and certain Tn3 members such as IS*1071*); ii) ISs with accessory genes not involved in transposition or regulation, which includes transporter IS and compound transposons; and iii) IS-related ICEs (IS-related Tpases employed for the integrating and excising of ICEs) and certain Tn3 members. The nonautonomous TEs (those lacking a Tpase and whose transposition requires the Tpase of a related element in the same cell) and TEs with passenger genes not implicated in transposition or regulation are reviewed elsewhere (282, 712).

The analysis of available genomes and metagenomes shows a limited distribution of most IS families among prokaryotes, with an over-representation of ISs among certain phyla, genera, and species (715, 786), which is probably associated with the exposure of such bacteria to variable, stressful, and new environments. Preferential IS occurrence is often observed for bacteria under adverse conditions, such as a challenge by antibiotics and other stresses related to contact with humans and animals (Enterobacterales and Lactobacillales), emerging species subpopulations and phylogenetically related pathogens with variable epidemiological and pathological features (e.g., the distribution of IS*4* among *Shigella* or *Xanthomonas* species, IS*431* [IS6] in *S. aureus* and skin microbiomes and ISCfe1 in *Campylobacter fetus*) (787), and bacteria living in isolated niches that limit the HGT of hosted ISs. A few major IS groups are predominantly involved in the capture or mobilization of ARGs, such as IS6/26 (IS26, IS257, IS1216), IS4 (IS10, IS50), and IS1111 (IS5), which are probably amplified by HGT events.

Due to the bias in the genomic databases, with overrepresentation of pathogenic and AMR strains, it is difficult to reach conclusions about the number and location of ISs, although there appears to be a preferential location within plasmids in antimicrobial-resistant bacteria (713, 715, 785). The number of copies also varies and is highly dependent on the

host, IS, and, indirectly, the "host range" of those ARGs. Barriers for IS uptake include uncontrollable transposition behaviors, lack of target site specificity, preferred insertions into essential genes and regulatory regions, and multicopy inhibition (788).

The effects of IS activity keep evolvability "on a leash". IS insertions can lead to the capture of antibiotic resistance genes in particular bacterial genomes. As emblematic examples, there are the members of the families IS*91* (789, 790), IS*6*/IS*26* (IS*26*, IS*257* and IS*1216*) and IS*ECp1* (in different families of Proteobacteria) (791–794), which are essential in acquiring and mobilizing a plethora of ARGs in Enterobacteriaceae, Staphylococci, Streptococci, and Enterococci, among others. IS insertions can also change the antibiotic susceptibility phenotypes toward either resistance or hypersensitivity by modifying the expression of antibiotic uptake determinants, transport processing, target sites, regulatory pathways, and efflux systems, eventually silencing genes/elements. For example, there is the increased resistance to fluoroquinolones after the insertion of IS*1* or IS*10* upstream from the *acrEF* efflux pump in *Salmonella* Typhimurium and the insertion of IS*186* upstream from the *acrAB* efflux pump in *E. coli*; the increased resistance to streptomycin after the insertion of IS*1133* upstream from Tn*5353* (*strA-strB*) in *Erwinia amylovora* and other species *(Forsters et al, 2015*); and the resistance to third-generation cephalosporins in *A. baumannii* after the insertion of either ISAba1 or ISAba125 upstream from the intrinsic beta-lactamase *ampC* of this species. However, ISs also determine the reversion of glycopeptide resistance of Tn*1546* operons toward silenced transposons in *Enterococcus* (712).

At the genome level, interactions between IS elements result in the generation of composite transposons. In addition to the classical examples of composite transposons involving members of the IS*4* (Tn*5,* IS*50*-Km-ble-str; Tn*10,* IS*10*-tet), IS*1* (Tn*9*) or IS*6* family (IS*26*, IS*257* and IS*1216*), a plethora of possible transposons can be generated

using subrogate ISs or subrogate ends (712). However, self-mobilization of these IS derivatives is influenced by the Tpase type and its orientation. The need to differentiate between mobile and non-mobile TEs (TEs vs. “pseudotransposons”) has recently been suggested (713). An important feature of IS-TE derivatives is their ability to provide a scaffold for recruiting new genes (791, 792), which can result in novel mobile composite platform variants (795) and select lineage-specific plasmid variants (796).

IS-mediated insertions and deletions can also result in changes in the genome structure, global cell regulation, and mutation rate of bacterial and plasmid backgrounds (797). The uneven occurrence of ISs is associated with the emergence of epidemiological or pathogenic variants at the species level (e.g., *Xanthomonas* species are enriched in different IS types) and subspecies level. Specific ISs are also linked to specific clonal pathogenic and AMR lineages (e.g., IS*1272* in CC29 *Staphylococcus haemolyticus*, ISCfe1 in *Campylobacter fetus)* (798) and are more abundant in human-adapted populations of various species, such as *Enterococcus faecium* (799, 800), *Enterococcus faecalis*, *S. aureus* (801), and *E. coli* (802). In the long term, significant genome-wide expansions were observed in only a few host-associated pathogens and in certain free-living extremophiles, suggesting that particular ISs could have been at least partially involved in the emergence or evolution of particular lifestyles, such as in *Bordetella pertussis*, *Yersinia pestis*, and *Francisella tularensis*. ISs influence the acquisition of exogenous DNA, including the inactivation of foreign plasmids and bacteriophages. In short, their activity constitutes one of the more important forces affecting the evolutionary trajectories of antibiotic/xenobiotic resistance in human and animal pathogens and, importantly, the trajectories of other MGEs and bacteria, favoring both the acquisition of resistance traits and constraints for the loss of genetic identity of the bacterial organism, maintaining “evolution-on-a-leash”.

ISs and IS-derived elements are themselves subjected to evolution, and their dissemination and maintenance has been explored theoretically (797). Transposition bursts are often interpreted as stress responses to environmental changes; however, the accumulation of stress events and elements would lead to unbearable fitness costs and possible extinction of hypertransposed populations following Muller´s ratchet-like processes, a type of evolutionary fatigue (803–805). Transposition bursts occasionally occur in the apparent absence of stress, as recently observed with ISs of the IS*30* family and the *mcr*-1 gene, which confers resistance to colistin (806). Such periodic transposition bursts assures the persistence of ISs in those populations (807, 808). ISs might also increase resistance expression, given that antibiotic stress results in IS activation by "activation complexes" formed by repressor-inhibitory mechanisms, a potentially adaptive mechanism, facilitating the insertion of ISs into sites that might allow the bacterium to survive antibiotic stress, resulting in a mutation-type strategy competitive with that of mutator genes (283).

Both insertions and deletions in the genomes where ISs reside are derived from "local hopping" and transposon immunity (809). Recent studies using *E. coli* as the targeted species have revealed that IS insertions occur 10-fold more frequently than IS-induced deletion events, despite the fact that deletions can vary under or in the absence of selection, implying that the genome tends to shrink without selective pressure (809, 810). Several explanations for IS dynamics using theoretical models have been offered (811–813). Maintenance of adaptive IS variants has been explained by three complementary hypotheses, focusing on IS selfishness (selfish DNA hypothesis), IS adaptive benefits (adaptive hypothesis), and IS adaptive neutrality (neutral hypothesis). These hypotheses explain the abundance of ISs in bacteria, which are influenced by drift, the frequency of HGT interactions, the positive or negative fitness effects of ISs, and, most importantly,

3410 the rate of transposition (808).

3411 **The Tn*3* superfamily.** Tn3-family transposons, classically known as "class
3412 II" transposons, are unitary noncomposite platforms that transpose by a replicative
3413 pathway, forming an intermediate cointegrate of donor and target molecules that are
3414 fused by directly repeated transposon copies. Classical Tn*3* members have three
3415 functional modules: a core transposition module that comprises a large transposase
3416 (TnpA) and an associated inverted repeat (IR), which are necessary for the cointegrate
3417 formation; a resolvase module (TnpR) with a serine or tyrosine recombinase; and a
3418 module of passenger genes. Most are autonomous elements with a complete
3419 transposition machinery that mobilizes the element in *cis*. However, a few Tn3
3420 composite transposons, pseudotransposons, and nonautonomous elements have also
3421 been described. Tn3 elements display transposon immunity, which precludes
3422 transposition of more than one copy of the element into a single replicon (814).

3423 The disparate phylogenies of the transposition and resolvase modules reflect a long
3424 coevolution that has resulted in a plethora of Tn*3* elements, typically classified according
3425 to the TnpA/IRs in large clusters that group TEs in disparate taxonomic groups, reflecting
3426 the general impact of HGT in MGE evolution and explaining the coevolution of TnpA
3427 and IRs to maintain specific and functional interactions between genetically connected
3428 hosts. Four large Tn*3* clusters are of special relevance in AMR, namely Tn*4430,* Tn*5393,*
3429 Tn*21*-mercury transposons, and Tn*3*.

3430 Tn*3*, which encodes blaTEM, was the first transposon described (originally named TnA)
3431 (815, 816) and was already widespread in early plasmids of various incompatibility
3432 groups (817) *Heffron (*(818) *and references herein)*. Mercury transposons have long been
3433 considered the flagship of AMR, because of the association of Tn*21* with class 1 integrons
3434 and other composite multiresistance platforms in early MDR isolates from the 1950s

(316). More recent studies have demonstrated a large diversity of mercury TEs in early AMR plasmids of human and environmental isolates, probably selected by the wide and intensive use of mercury in the early part of the 20th century. These transposons would have subsequently and independently acquired class 1 integrons (819). Emblematic examples of Tn*3* mercury members include Tn*21*, Tn*1696,* Tn*501,* and Tn*6182,* all globally distributed in epidemic plasmids or embedded within resistance islands (820–822). Tn*4430* includes TEs widely spread in the staphylococci and/or enterococci Tn*917* (*ermAB,* encoding erythromycin), Tn*551* (*bla,* encoding the beta-lactamase), and Tn*1546* (*vanA,* encoding high-level resistance to glycopeptides). Another group represented by the emblematic Tn*5393* (*strAB*), present in all plasmids recovered in the 1950s and clustering other similarly cryptic TEs such as Tn*5403* and Tn*3434*, was initially found in the environment and is now increasingly associated with mobile composite elements, including the bla_{KPC} and bla_{NDM} genes (823, 824). This group also helps other MGEs; indels and rearrangements are frequent and appear in both contemporary and early plasmids. Composite elements including Tn*3* are apparently exceedingly rare, because transposition immunity precludes transposition of more than one copy of the element into a single replicon. Pseudotransposons and nonautonomous elements related to Tn3 have been described.

The Tn*7* superfamily. The Tn*7* superfamily comprises unusual, highly sophisticated and extremely efficient MGEs, which are characterized by their transposition machinery (a core of three transposition proteins [TnsABC(R)] and two target selection proteins [TnsD(Q) and TnsE]) and by displaying, in addition to Tn*3*, transposon immunity (825). Tn*7* frequently targets an *att*Tn*7* chromosomal site (*glmS* gene), an essential gene conserved in highly divergent bacteria. This propagation occurs in a neutral manner and leads to the successful propagation of adaptive traits by vertical

transmission (through TnsABC+D). Tn*7* also targets conjugative plasmids and bacteriophages at a low frequency (through TnsABC+E). There are strategies that relax the target specificity, as well as alternative target locations, including interactions with other MGEs, such as MICs and genomic islands (826–829). Remarkably, these elements are the main vehicles of class 1 (Tn*402*) and class 2 (Tn*7*) integrons (717, 830–832).

According to the phylogeny of the transposases, Tn*7* elements are classified into three groups: Tn*7*, Tn*5053*/Tn*402*, and Tn*552,* each with a GC content that reflects the preferred bacterial host and thus an ancestral adaptation to distinct prokaryotic groups (826). There have been an increasingly large number of reported **Tn*7* variants** carrying genes coding for resistance to antibiotics (embedded in class 2 integrons, genomic islands, and IS-related TEs), heavy metals (operons or clusters associated with silver, copper, and chromate resistance) (827, 828), and CRISPR or RM systems, among many other adaptive traits (826, 833), prompted by IS-mediated homologous recombination.

Tn*5053*/Tn*402*-like transposons. Tn5053/Tn402-like transposons (TniABQR) have target preference for the *res* site of plasmids and TEs of the Tn*21* subfamily and therefore are known as "*res* hunters". Resolvases (*res*) function to resolve plasmid dimers following plasmid replication. Tn*5053* are predominant in disparate environmental settings, and occasionally in clinical isolates of *Pseudomonas* (e.g., Tn*502,* Tn*503*); however, Tn*402* elements are distributed in many prokaryotic groups associated with various hosts. A plethora of Tn*402*-like transposons have been reported, including variants with defective tni_{Tn402}, class 1 integrons (834, 835), and hybrids of Tn*7* and Tn3 (Tn*5053/*Tn*402*; Tn*21*/Tn*501*), which would have spread via HGT and recombination with many different MGS (829).

Tn*552*-like elements. Tn*552*-like elements encode the beta-lactamase genes of staphylococci from their early spread after the drug's therapeutic introduction. These elements are extremely frequent in multiresistance plasmids typically inserted within the *res* site of the plasmid's resolution system. In many cases, genetic rearrangements are evident within or in the vicinity of these elements, presumably mediated by interactions between the transposon and plasmid resolution systems and repeated transposition events into the elements.

Nonautonomous Transposable Elements. Nonautonomous TEs are fully dependent on trans-acting compatible transposases encoded by related functional (autonomous) TEs and include small (generally less than 300 bp) elements, such as miniature inverted-repeat transposable elements (MITEs) and mobile cassettes (MICs), whose transposition can be catalyzed *in trans* by a transposase of a related IS (712, 836). MITEs greatly contribute to the spread of antibiotic resistance from environmental species into *Acinetobacter* (837, 838), Enterobacteriaceae (839), and *Aeromonas* (840) bacterial families.

MITEs, and repetitive extragenic palindromic elements are small, nonautonomous IS derivatives whose transposition can be catalyzed *in trans* by a transposase of a related IS (712, 836). These elements are represented throughout the microbial world, indicating an ancestral origin for these sequences. A linear correlation between IS and MITE abundance has been observed, such as the conserved 439 bp MITE-like structures flanking integrons found in *Acinetobacter* species of disparate origins that facilitate the acquisition and spread of various beta-lactamases (838, 841) the integron-mobilization units carrying *bla*$_{\text{GES-5}}$ located on plasmids of *Enterobacter cloacae*; and others found in plasmids or in either *Enterobacterales* or *Acinetobacter* (837, 842). Tn3-derived inverted-repeat mobile elements are specialized MITEs (843, 844), which can regulate the expression of genes by insertion within protein coding sequences and are responsible for the mobility of

antibiotic resistant class 1 integrons located in both plasmids and chromosomes (841, 842). Different IS families show target specificity for repetitive extragenic palindromic sequences (IS*3*, IS*110*, IS*4*, IS*256* and IS*5*), which is not surprising, given that the features of the DNA target and of the transposase domain responsible for target choice are not included in the criteria for defining IS families.

Genomic islands. Genomic islands (GIs) are large, continuous genomic regions of variable size (4.5–600 kb) engendered by HGT (and thus with a different GC of the core genome) and heterogeneously distributed within prokaryotic groups (845–847). Among GIs' most relevant features are the presence of mobility-related genes (*int* and xis, transfer origins, *tra* genes, replication-related genes, and transposition genes), flanking direct repeats, and specific integration sites. Thus, the "island family" composite platforms include MGEs, ICEs, pathogenicity islands, resistance islands, symbiosis islands, integrating plasmids, and probably prophages. Most prokaryotic groups have different types of genomic islands (731, 848).

GIs play a relevant role in microbial genome evolution and adaptation of bacteria to environments, often in quantum leaps, allowing bacteria to gain large numbers of genes related to complex adaptive functions in a single step, thereby conferring evolutionary advantages. For example, GIs of Staphylococci (SaPIs and SCCmec) (731), *Vibrio cholerae* (SXT/391, other GIs) (848), *Salmonella* (SGIs), *Acinetobacter* (AcRo), and *Proteae* (PGIs) can be mobilized by plasmids (837, 849, 850) or phages (731).

Integrative-conjugative elements, ICEs, are modular autonomous GIs that share similarities with conjugative plasmids (conjugation) and viruses (integration and excision), are widely distributed and are probably more common than plasmids (851). Most of the available information on ICEs comes from comparative genomic analysis, revealing gene content, functionalities, and evolutionary history (852). Certain ICE

families have been characterized in detail, especially those associated with antibiotic resistance, such as SXT/R391 (MPF_F type), Tn*916* and ICEBs1 (MPF_{FA}), and CTnDOT (MPF_B). These cases show that ICEs have greatly influenced the fitness of pathogenic (and probably also drug-resistant) bacterial lineages (853).

GIs share alternate states of integration, excision, and transfer, although the regulation of these states varies greatly among elements. The different requirements for the integrated and excised forms of GIs/ICEs now suggest the inability to coexist in the same cell and have led to the hypothesis that most ICE systems go through a bistable activation state, followed by ICE excision of a dedicated subpopulation and possibly by a dedicated transfer competence development program (854, 855). The bistability hypothesis helps to understand the lifestyle of ICEs, including the relationship with the host and the selective forces behind their vertical and horizontal transmission modes. According to this hypothesis, programmed regulatory networks would indicate that only a small specific subpopulation (coincidental with the variable transfer rate of these elements; e.g., 10^{-2} to 10^{-7}) is able to excise. The small size of this excisable and eventually "transferable" population is explained by high cost that would be invested in the transfer event. Major strategies to assess the stability and maintenance of certain GIs include limited replication, deployment of active partitioning systems, and the active killing of donor free cells due to either an abortive toxin-antitoxin (TA) infection system or a novel mechanism only observed in the SXT/391 family, the so-called "trap-door". Recombination between elements occurs if they do not belong to similar exclusion clusters.

Bacteriophages and phage-related particles. Bacteriophages are the most abundant type of microbe, with an estimated number of 10^{31} phage particles worldwide (856, 857). Bacteriophages depend on bacterial cells for propagation and are therefore key drivers of bacterial population density, constantly promoting their own diversification and the

diversification of their bacterial hosts, which has evolutionary consequences that have not yet been fully explored. Phages contribute to clonal oscillatory dynamics in the host microbiota, helping the spread of the best colonizers (given that phages frequently carry colonization-virulence factors) and high-risk resistant clones (given that they probably contribute to non-host-derived immunity) (858). Bacterial lysis by phages should release free DNA (including resistance genes) into the environment and might contribute to gene spread by transformation in natural habitats (859). The influence on phylogeny (e.g., the emergence of clones or clonal ensembles) of DNA transfer by phage transduction depends on species-phage specificity; lysogenic or temperate phages tend to have greater specificity than lytic phages (860). Temperate phages, integrated into the bacterial genome, are probably one of the more efficient agents of HGT (transduction). Transduction events occur up to an estimated 20 x 10^{15} times per second (857, 861). Antibiotic exposure can activate the lysogeny of temperate phages, eventually favoring the transduction and expression of phage-contained virulence genes (862).

Transduction can result in the transmission of chromosomal host genes carrying resistance mutations, mistakenly integrating them into the phage genome. The role of bacteriophages and phage-related particles as reservoirs and drivers of AMR in the human and animal gut, sewage, and agricultural soils has been extensively studied (863–867). Mobilization of chromosomal AMR genes by transduction has been demonstrated for major opportunistic pathogens such as *Enterobacterales* (*E. coli* and *Salmonella*), although much more frequently in streptococci and staphylococci. In the latter cases, antibiotics at subclinical concentrations have been shown to promote the bacteriophage transduction of ARGs.

Why have so few AMRs been reported to be present in phages compared with plasmids? A first comparison of network properties between plasmids and phage genomes revealed

that plasmids are more frequently connected within the bacterial network compared with phages. Conjugation is thus more frequent than transduction in nature (868), with a transduction/conjugation rate of approximately 1/1000 (862). The bacteriophage host range could be narrower than plasmid promiscuity, resulting in fewer captured "genome externalized genes", probably 10 times less frequently than plasmids. However, gene flow between MGEs occurs preferentially between consistent groups of genomes; for instance, phages with phages and plasmids with plasmids (869). Chromosomal ARGs are infrequently located in core genome regions, which are the common sites of prophage integration. The frequency of specialized transduction events carrying ARGs is estimated at approximately 10^{-9} transductants/plaque forming units but can be higher in *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium* (862), which correlates with the higher frequency of ARGs transmitted by phages in these taxons. Moreover, the cost of carrying antibiotic resistance genes might restrict phage evolution (870). When CRISPR-Cas immunity toward foreign DNA is borne by lytic phages, the host bacteria are prevented from acquiring plasmids, eventually carrying resistance determinants. Evasion of CRISPR immunity by plasmids occurs at the host level through high frequency loss of functional CRISPR-Cas immunity at a frequency as high as 10^{-4} in the case of the conjugative plasmid pG0400, which encodes mupirocin-resistance. However, CRISPR can be reacquired by HGT in environments where phages are a major cause of mortality (871).

Phages can combine with other MGEs, such as plasmids, transposons, and genetic islands, forming phage-like elements (865). One class of phage-like elements, called gene transfer agents, is based on the presence of usable capsids in the bacterial chromosome, facilitating mobilization of bacterial DNA (872), which can transfer antibiotic resistance in heterologous recipients at higher frequencies than previous estimates of their

transformation and transduction rates in natural environments (10^6-fold higher). The host range, however, appears to be very concentrated in alpha-proteobacteria from ocean environments. Ecological co-occurrence with pathogens is needed to create a significant risk of AMR acquisition by phages and phage-related elements (772, 860).

Flow of Mobile Genetic Elements and Antimicrobial Resistance Genes

Most AMR genes are "mobile" because of MGEs. The term "mobile" here indicates the ability of being transmitted among heterogeneous biological entities. However, the term "mobile" or "mobilization" has another semantic value, the one used in economics, law, and communication sciences: "to bring (resources or reserves) into use for a particular value" (873). Mobility has a *raison d'être*; i.e., it creates value for both AMR genes and bacteria and for the microbial community acquiring the genetic trait. To play a significant role in ecology and evolution, the "value" created by the genetic transfer system, which provides adaptive advantages (in our case, antibiotic resistance), should be based on the robustness (ability of the system to tolerate irregular changes) and conduciveness (efficacy in reaching the goal of resistance) of the players facing different ecogenetic contexts. These advantages create "highways" where AMR genes are maintained and circulate in a consistent, sometimes permanent manner. These facilitated processes are frequently derived from the historical biological background of genetic exchanges and conditioned by the ecological continuity required for continuous mobility.

The environmental context of antibiotic resistance gene flow. Limitations in the availability of adaptive DNA and MGEs and transfer-proficient bacterial subpopulations determine the possibilities of antibiotic resistance determinant mobilization, which is influenced by environmental disturbances. Fluctuations in the environment are heterogeneous, irregular, and often stochastic. The resistance and resilience of a functioning ecosystem depends on the species' richness; in the case of antibiotic

resistance, the more MGE and subpopulation diversity, the more chances to respond to irregular and sudden perturbations, increasing the AMR evolvability (746). The primary benefit of bacterial diversity would be to acquire robustness to face sudden and uncertain challenges, such as antibiotic resistance. However, the number of variants that generate robustness can vary during evolution due to the low or infrequent temporal occurrence of the changes. Thus, the balance between robustness and evolvability drives the evolution of antibiotic resistance entities (874). A major source of environmental variation derives from anthropogenic activities, which are increasingly considered in the analysis of antibiotic resistance under the One Health, Global Health, and Planetary Health perspectives (875, 876).

Cell-free DNA as a source of ARGs has increasingly been reported at the interface of the human and water environment (877). Depending on the bacterial species involved and the gene-transfer mechanisms that are active, a number of processes limit (or enhance) the transfer, uptake, and stabilization of foreign DNA in bacteria from different environments. The canonical HGT mechanisms of conjugation, transduction, and transformation involve genetically and ecologically connected populations (313, 325, 851, 868, 878, 879). Other HGT mechanisms are increasingly being documented in soils and marine habitats, such as DNA-packing extracellular vesicles and DNA transfer through intercellular nanotubes (880–883). Extracellular vesicles coordinate numerous forms of intercellular communication and facilitate the exchange of small molecules, proteins, and nucleic acids, including RNA and DNA and elements such as plasmids. Interspecies vesicle-mediated gene transfer has been reported in *E. coli*, *Acinetobacter baumannii*, *A. baylyi,* and *P. aeruginosa* (884, 885). The combination of various HGT processes is now recognized as a primary strategy for transmission and cooperation

between natural bacterial communities in order to exploit genetic common goods, such as ARGs (886).

Highways for antibiotic gene flow vary according to the environmental factors, which have dramatically changed during the 20^{th} century due to massive anthropogenic interventions. The release of antibiotics, heavy metals, pharmaceuticals, and manure into the soil and water ecosystems is expected to greatly affect the composition and dynamics of resistomes and HGT events in nature because they provoke acidification/ pH changes and the introduction of organic matter and exogenous DNA. Major molecular effects from these stressors include triggering the SOS response, increasing reactive oxygen species levels, weakening the cell wall, modulating quorum-sensing processes, increasing adaptive antibiotic resistance, and enhancing HGT (325, 348, 887–889). The transient bacterial communities composing manure soils imply that transformation or phage transduction (also present in these environments) could have a relevant role (890). Kotnik and Weaver have estimated that, under contemporary ecological conditions, at least 10^{24} microorganisms are subjected to a freeze-and-thaw cycle, at least 10^{19} are subjected to sand agitation, and at least 10^{17} are subjected to conditions suitable for electrotransformation in any given year. Common minerals employed in animal food supplements and biosolids promote the direct transfer of antibiotic resistance plasmids between bacterial species (891, 892). Most species involved in antibiotic resistance are generalist and are thereby able to cross different host species (893). The conduciveness of ARGs depends on MGE promiscuity, which is determined either by ecological opportunity (plasmids and other conjugative elements) or phylogenetic distance (bacteriophages). Each element employs preferential transfer mechanisms in which recipients and donors play different roles, determining preferential roads for antibiotic dissemination. Changes in reservoir size and in ecotones can facilitate the emergence and

persistence of pathogens and the antibiotic resistance traits they carry, as has been reported for MRSA (894) enterococci (895) and other organisms.

Gene flow and DNA uptake proficiency. Recipients play a central role in natural transformation. Naturally (heritable) occurring bacterial subpopulations with enhanced competence or recombination potential (mutator strains) have been associated with ARGs and MGEs in the various species frequently involved in antibiotic resistance (896). Competence development is often explained by the phenomenon of phenotypic bifurcation or "bistability", traditionally interpreted as stochastic events triggered by environmental stimuli that now appear to be highly regulated processes within individual cells (897, 898). Environmental distribution and dynamics of mutator phenotypes is still unknown.

The recombination of homologous or heterologous acquired DNA has been extensively revised elsewhere (879). The contribution of DNA uptake in natural environments appears to have been greatly underestimated. The acquisition of transposons, integrons, and gene cassettes by competent disparate species (899) and the possibility of acquiring large fragments and antimicrobial-resistant genes (900) frequently occurs. Recent studies that relate competence for killing nearby cells via fratricide or sobrinicide (in *Streptococcus*) or by kin-discriminated neighborhood predation (through T6SS systems in *Vibrio* and *Acinetobacter*) have revealed an active HGT strategy for acquiring exogenous DNA that can contribute to the fitness of the predator after acquiring beneficial adaptive traits, including the uptake of plasmids (901–903). Co-regulation of competence and T6SS systems, described in *Vibrio cholerae* and *Acinetobacter*, could be important for other genera involved in AMR uptake, such as *Campylobacter*, *Pseudomonas*,

Agrobacterium, and *Ralstonia.* Lastly, transformation has recently been suggested as a relevant process to rescue bacterial cells from selfish mobile elements (904).

Gene flow and conjugation proficiency. Donors play a central role in conjugation, whereas recipients often limit the transfer or the establishment of the conjugative elements. Transference is highly regulated in plasmids and differs between Gram-positive and Gram-negative species (comprehensively revised in #Kohler 2019 and references herein). Despite the differences in backbone, regulatory networks, and evolutionary origins, ICEs appear to have a relatively restricted host range and share a general model of bistability that explains their horizontal or vertical transmission (855, 905). Conjugative elements frequently interact with other elements within the cell (see plasmids for some emblematic examples) and can modify the HGT ability in recipients (904, 906). MGE promiscuity is related to this affinity requirement and to the availability of attachment sites in the recipient. Hotspots for a specific insertion site are common for biologically relevant GIs, transposons, and bacteriophages in species of Actinobacteria, Firmicutes and Proteobacteria (e.g., the 3' end of the housekeeping gene glutamine aminotransferase [GMP synthetase]), although such specificity can be relaxed, facilitating uptake at secondary sites ((907) and references herein).

Gene flow and the acceptability to foreign genes; defence systems. Depending on the bacterial species and MGEs involved and the gene transfer mechanisms, a number of processes limit (or enhance) the transfer, uptake, and stabilization of foreign DNA molecules in bacteria. Recipients already carrying conjugative elements limit the acquisition of similar entities by incompatibility (plasmids) and exclusion (plasmids and ICEs). Plasmid incompatibility is often modified by recombination, which explains the frequent coexistence of similar plasmids in antibiotic-resistant bacteria, such as F

plasmids in *E. coli* and pheromone-responsive plasmids in *E. faecalis* (729, 772, 908). Incompatibility also affects the dynamics of ICEs and plasmids with the same replication machinery (909). Surface/entry exclusion affects plasmids and ICEs of differing GC content (910). Whereas surface exclusion prevents close contact between cells, entry exclusion prevents DNA transfer after the formation of the mating pair.

Defense systems prevent the introduction of heterologous DNA from conjugative elements and phages and are classified into two major groups, namely immunity and dormancy induction and programmed cell death, which can be collected, analyzed, and visualized in a comprehensive prokaryotic antiviral defense system database comprising elements from more than 30,000 species (*https://bigd.big.ac.cn/padsarsenal*). The immunity group includes RM systems, bacteriophage exclusion systems, and clustered, regularly interspaced, short palindromic repeats adjacent to *cas* gene (CRISPR-Cas) systems. The dormancy induction or programmed cell death by the infection group includes TA systems and abortive infection (911, 912). Defense mechanisms show nonrandom clustering suggestive of nonadaptive evolution of the islands through a preferential attachment-like mechanism underpinned by addictive properties (913), which can eventually act as selfish mobile elements.

Barriers between different prokaryotic groups and antibiotic resistance gene flow. Phylogenomic networks employing genomes and metagenomes reflect the major impact of HGT during microbial genome evolution, suggesting barriers at multiple levels between various prokaryotic groups (914–916). Phylogeny correlates with ecology, the field of eco-phylogenetics, and phylogenetic community ecology (917). If ARGs are expected to exist virtually everywhere, consistent with the Baas-Becking principle (918), they are selected and circulate and evolve preferentially among phylogenetically related organisms not only because of their ecological coincidence but also because they have

been evolutionarily adapted to the genetic background and physiology of groups sharing a common ancestor. Genes recently acquired via HGT are more similar in codon usage than the genes that have been vertically inherited (919); for instance, recently acquired genes tend to be relatively AT-rich compared with the host's chromosome. The phylogeny of RM systems also correlates with the phylogeny of the bacterial taxons; these mechanisms against foreign DNA create preferential pathways of genetic exchange, within and between lineages, with related RM systems (920). Transferred genes are concentrated in only approximately 1% of the chromosomal regions (315), and the density of chromosomal hotspots for integration of foreign genes in different species should therefore influence the acquisition of ARGs.

However, HGT occurs at a lower frequency across diverse bacterial phyla (921) linking distinct genetic pools (868, 922, 923). Barriers to HGT between distantly related bacterial species (having dissimilar genomes) are still poorly understood but are thought to depend on the transfer mechanism (broad host range MGEs) and community permissiveness, which refers to a community's ability to share a gene acquired by HGT (genetic exchange community). Ecologically cohesive bacterial populations forming a multispecies community (coexisting in biofilms) should have better chances to establish a "common good", assuring the resilience of the community partners involved in cooperative functions (924). The analysis of networks focused on genes shared between chromosomes of different species, plasmids, and phages shows that not only genes are preferentially shared between groups of closely related genomes and between typologically consistent groups, as phages with phages and plasmids with plasmids (869) but most gene transfers occur within particular geolocalized habitats (78, 742). However, ecologically isolated populations (including many intracellular bacteria and those tolerating unique stressful environments), which are also in genetic isolation, are less prone to receiving ARGs

(545). Co-operative or competitive-amensalistic interactions between species should influence co-occurrence at short distances and HGT. Recent genomic and metagenomic developments should cast some light on the complex field of ARG flow (925).

Barriers determined by the interactions between mobile genetic elements

Interactions between coexisting MGEs are common. Most bacterial pathogens host a multiplicity of potentially interacting MGEs (752), obtained by sequential or simultaneous acquisition or by long-term local plasmid evolution. These interactions can alter, among other things, MGE transferability and maintenance. Mobilizable plasmids, which comprise at least 25% of all plasmids, rely on other conjugative elements present on the host cell to be able to spread by conjugation (926, 927). Conjugative plasmids might also facilitate the conjugation of another conjugative plasmid present in the cell, a phenomenon that frequently involves plasmid-plasmid RecA-dependent cointegration, sometimes using common transposable elements (such as IS*26* in carbapenemase-carrying plasmid cointegrates) (928). However, facilitation of the transfer of a co-resident conjugative plasmid does not necessarily involve conventional RecA-dependent recombination. Facilitation is negatively influenced by the surface/entry exclusion but enhanced by favoring donor-receptor "mating clumps" mediated by plasmid-encoded sex pili (929, 930). MGEs affect the fitness effects produced by other MGEs coexisting in the same cell. Plasmids, for example, typically engender a fitness cost in the host bacterium (749, 931) however, these costs can be ameliorated (positive epistasis) or accentuated (negative epistasis) by the presence of additional MGEs (752, 932). Epistatic interactions between MGEs can determine the fate of the MGE in bacterial populations, promoting low-fitness-cost associations and long-term maintenance, thus shaping the highways of AMR genes (752, 933, 934). Plasmid evolutionary success and the plasmid-mediated spread of AMR are to a significant degree the result of a intracellular plasmid competition

with other plasmids, influencing the spread by lateral transfer, in particular, the stable plasmid inheritance (incompatibility) (935). Conjugative plasmids commonly encode fertility inhibition determinants, which reduce the conjugation frequency of other plasmids present in the same cell (936). Plasmid incompatibility is based on common regulatory mechanisms of coexisting plasmid replication, resulting in a competitive replicative dynamic leading to the loss of one of the plasmids in the cell progeny. Replicon typing has served as a method for classifying plasmids (*Inc* or *Rep* typing) (937). However, there are numerous examples in natural bacterial isolates of incompatible low-copy-number conjugative plasmids carried jointly, providing evidence that resistance plasmids can solve incompatibility, increasing the cellular repertoire of ARGs (930, 938). Incompatible plasmid coexistence can result from cointegration or from plasmids harboring more than one mode of replication (768). Plasmid localization and partition (Par) systems also cause plasmid incompatibility, such that distinct plasmids with the same Par system cannot be stably maintained in the same cell (939). In addition, TA systems can eliminate incompatible plasmids from the progeny (940).

There should be a vast number of continuous interactions between MGEs in single cell progeny, including phages. A fascinating example of how interactions between MGEs affect their horizontal transmission is the arms race between phages and pathogenicity islands in *Staphylococcus aureus*, in which both elements compete using a complex repertoire of molecular interactions packaged in the phage capsid (941). Other exemples of interactions among MGEs occurs among pipolins, self-synthesizing transposons encoding replicative B DNA polymerases), which can be present in *E. coli*, but not involved in antibotic resistance, and other integrative MGEs as integrons (942).

Mobile genetic element dispersal within species. The concept of species remains elusive in bacteriology (943, 944). In the age of whole genome sequencing, it is widely

accepted that strains belong to the same species if they share more than 95% average nucleotide identity. Although MGEs belong to the accessory genome and considering there is a common evolutionary history for MGEs and their usual hosts, a mutual adaptation has taken place. However, many plasmids (more than 50% of those examined by bioinformatic methods) are able to colonize species from different phyla (716). In any case, it remains true that the same type of MGE tends to be associated with the same type of host (945). Historical coexistence with MGEs has likely contributed to speciation (or at least with the gene regulatory mechanisms that impose "styles of life") in a particular ancient host (946). It is not surprising that mobility and maintenance should be more effective within particular speciesPlasmid stabilization is likely to occur in a bacterial host, mediated by different mechanisms, such as mutations in a replication protein gene, acquisition by the resistance plasmid of a transposon from a co-residing plasmid encoding a putative TA system, and a previous mutation in the host's global transcriptional regulation genes (551). The process of stabilization by mutation of the plasmid replication protein involves the emergence of numerous plasmid variants differing in this initiation protein; clonal interference (competition between variant clones) thereby determines the evolution of the persistence of drug resistance (947). Plasmid-encoded TA systems have an advantage in within-host plasmid competition if the host cell is sensitive to the toxin (948). Long-term coevolution of a plasmid in a particular species can result in partially or fully codependent replicons, a "plasmid specialization in particular species", limiting the spread to other lineages in which the maintenance or expression of plasmid traits, such as ARGs, could be reduced (748, 949).

Most bacterial species tend to diverge into subspecies and clones by the process of "clonalization" (mimicking speciation by adaptation frequently mediated by HGT) to neighboring ecological niches (ecovars). This ecological neighborhood facilitates the

evolution of plasmid-host specificity, frequently overcoming the process of clonalization (950). Indigenous MGEs thereby contribute to the communal adaptive gene pool of the species. This resilience of the plasmid-host specificity pattern involves regulation of the defense mechanisms that might be present in the species (951).

Intracellular dynamics of mobile genetic elements. MGEs such as ISs and transposons can move almost randomly (sometimes with associated ARGs) from one location to another within the genome (chromosomes or plasmids) of a bacterial cell. Integrons employ site-specific recombination to transfer resistance genes between defined genomic spots. An average of 10^{-4} IS insertions and 10^{-5} IS-mediated recombinations per genome have been estimated per generation in the *E. coli* K12 genome (952). How are ISs maintained successfully in bacterial organisms despite transposition bursts frequently being deleterious to their host genomes, often induced by stress, including antibiotic exposure? The intake of ISs through the uptake of MGEs is insufficient to replace lost ISs; however, continuous adaptive genetic variation resulting from insertion events can be maintained as "evolutionary insurance" for bacterial adaptation to changing environments, which could facilitate homologous recombination, removal of deleterious genes, and acquisition of advantageous mutational events (808), as well as ensuring crosstalk between genetic regions of the cell, sometimes from different intracellular replicons. Most importantly, ISs (and composite transposons) are associated with the acquisition of ARGs (953). In a section above, we mentioned the role of ISs in keeping evolvability "on a leash". ARG gene shuffling is a consequence of intracellular MGE mobility (954).

Integrons are extremely ancient groups of elements with low basic diversity (only three main classes associated with broad bacterial taxons but with many variants) and widespread chromosomal elements. Integrons are not MGEs in their own right, given that

the integron integrase cannot excise its own gene from a chromosome; however, integrons can gain mobility (mobile integrons) through intracellular association with transposons or plasmids and can carry ARGs (955). For instance, integrons can be inserted at different locations into distinct ancestral transposons, such as mercury transposons (820, 956). Integrons also act to efficiently capture exogenous genes ("adaptive on demand" genes, including antibiotic resistance genes) that are acquired (and excised) as "gene cassettes", expressed under the function of an external promoter. It is unclear how genes that originate in different species and environments reach and are recruited by the integron; however, the acquisition of mobile integrons carried by plasmids or mobile transposable elements could play a relevant role (957). The order of gene cassettes in the string (possibly hundreds) can be changed, thereby altering the distance to the promoter (717). Mobile promoters can be horizontally transferred (718) and can sometimes influence the expression of antibiotic resistance genes by intragenomic mobility (958). MGE dynamics is regulated by the cell to reduce "intragenomic conflicts" (959), ensuring a "maximum tolerated number of copies, from plasmids to transposable elements, as occurs in transposon immunity (960).

Intracellular interactions between plasmids and the chromosome also constitute a relevant topic. Hypothetically, the translocation of these genes from the plasmid to the chromosome, followed by "costly" plasmid loss in the progeny could keep the advantageous genes carried by a plasmid without the cost of maintaining the replicon (961). However, plasmid loss is frequently minimized by compensatory evolution, and the process of antibiotic resistance gene capture by the chromosome occurs infrequently. (962). In principle, small plasmids with a high number of copies per cell should be more difficult to eliminate than large plasmids with a small copy number. A debatable issue is whether small plasmids impose a different fitness cost than large ones; however, meta-

analysis studies have suggested that there is not much difference. The fitness cost is proportional to the number of antibiotic resistance genes carried in the plasmid, suggesting that plasmid loss should be more frequent in multiresistant plasmids (934).

The intracellular evolution laboratory for antibiotic resistance. We have highlighted the multiple, almost unlimited wealth of intracellular interactions among MGEs and with the bacterial chromosome, which creates a scenario of overwhelming complexity, in which a multiplicity of genetic combinations is constantly created and offered to natural selection in various environments. These experimental combinations can surpass the normal mutation rate and can also impose a lower fitness cost for the cell in the medium and long term. Plasmid carriage has a lower average fitness cost than chromosomal mutations (934). The fuzzy ontology of MGEs, where the interaction among phages, plasmids, and transposons produces a "mosaic continuum", provides an accurate image of this "intracellular evolution laboratory" (963). A good example is the unpredictable structure of mosaic plasmids, composed of genetic elements from distinct sources; approximately 50% of plasmids represented in databases are in fact mosaic plasmids, unevenly distributed across bacterial taxa, although possibly more common in more environmentally connected species (964). The genetic diversity of mosaic plasmids has contributed to the selection and spread of antibiotic resistance (908, 965) but has increased entropy while predicting evolutionary trajectories.

The Ecogenetics of Antibiotic Resistance Transfer and Maintenance: Antibiotic Resistance Genes in the Accessory Genome

MGEs should be transferrable from a donor to a receptor bacterial host, a transfer that depends on the autonomous ability of the MGE to encode its own transfer mechanisms

or to be mobilized *in trans* by another MGE. The transfer event will have little to no functional consequences in the absence of MGE compatibility with the host genome, including the host-resident MGEs. MGE mobility among bacterial organisms does not ensure the expression of the ARGs they might carry in the recipient cell. A resistance gene present in an MGE might also persist in the recipient cell (by recombination in the host genome) even if the MGE is rejected. Most transmissible ARGs should correspond to the "accessory genome", and the trajectories of genes belonging to the mobile accessory-adaptive genome should correspond to ARGs, which is illustrated in Figure 7 and detailed below. In this section, we discuss the mobility of antibiotic resistance based on resistance genes, as part of the mobile accessory or adaptive genome.

Trajectories of accessory genome genes in Gammaproteobacteria. The gene flow trajectories in Gammaproteobacteria are clearly related to the species' phylogenetic neighborhood. Accessory gene flow analysis among Gammaproteobacteria reveals a "core ensemble of species" in Enterobacterales, constituted by *Escherichia*, *Klebsiella*, *Salmonella*, *Citrobacter*, and *Enterobacter*, followed in descending order by *Serratia* and *Yersinia*, *Pasteurella*, *Haemophilus*, *Vibrio*, *Acinetobacter*, *Pseudomonas,* and *Legionella* (514). These accessory gene exchange ensembles correspond closely to the Enterobacterales' phylogenetic groups (966). In principle, accessory (and resistance) gene spread should be facilitated among members of the same phylogenetic ensemble, such as *the Escherichia-Enterobacter* clade, composed by *Escherichia, Klebsiella, Enterobacter, Raoultella, Kluyvera, Citrobacter, Salmonella, Leclercia, and Cronobacter*. Other Enterobacterales clades include *Erwinia-Pantoea*, *Pectobacterium-Dickeya*, *Serratia-Yersinia*, *Hafnia-Edwardsiella*, *Proteus-Xenorhabdus*, and *Budvicia.* Ecological distancing affects bacterial interactions, and an eco-phylogenetic approach might be established to predict significant gene flow. To define such trajectories, it is

important to analyze the health risks of the emergence of a particular antibiotic resistance gene in a particular species.

Accessory genome trajectories in Firmicutes

In Firmicutes, the accessory genome clusters are more dispersed than in Gammaproteobacteria. Stronger interactions are found among a core of *Streptococcus*, *Enterococcus*, and *Staphylococcus* clusters and weaker interactions are found with *Clostridioides*, *Bacillus*, *Clostridium*, *Lactobacillus*, and *Leuconostoc* clusters. However, all of these clusters share accessory genes and, potentially, ARGs. The structure of these interactions fits well with the protein content network of antibiotic resistance proteins found in the plasmids and chromosomes of Firmicutes (967). As in the case of Gammaproteobacteria, gene flow is highly dependent on the ecogenetics of the various species (e.g., *Listeria*, which, despite being located in the vicinity of the *Streptococcus-Enterococcus-Staphylococcus* exchange cluster, undergoes infrequent acquisition of accessory genes and resistance genes from phylogenetically related species).

Evolutionary Kin Hindrances and Shortcuts: the Role of Relatedness

How does relatedness between bacterial lineages influence linked evolutionary processes? In a certain sense, the evolutionary success of a member of a given lineage group *is* the success of this group in competition with other groups. The winner, typically the best-adapted clone, was probably positioned by previous successes of the group in the circumstances that facilitated its own selective advantage, a feature that can be considered as a “group investment” in the success of one of its members. This investment should now produce a return for the benefit of the winning kin-related members of the group. Ultimately, the evolutionary advantage frequently benefits the entire group. How is the

evolutionary benefit re-distributed? If the winner protects the whole group by producing molecules protecting from antibiotics the bacterial ensemble then HGT plays a major role here. The winner increases in population size and redistributes the acquired trait among the kin-members (relatives) of its group.

Facilitated recombination between gene families. Recombination, the biological process by which two genomes exchange DNA sequences, is a fundamental evolutionary process that has profound effects in bacterial genomes. Recombination creates chimeric genomic sequences and can unite beneficial genes (or mutations) that emerged separately (968). Recombination is responsible for spreading ARGs across bacterial populations (969). However, not all genomic sequences are equally likely to recombine. Recombination requires short segments of nearly identical DNA sequences flanking the genomic regions to be exchanged. The minimum length of these segments varies depending on the species but is typically in the range of 20–100 nucleotides (970, 971). The probability of finding nearly identical sequences decreases with genomic divergence; thus, recombination occurs more frequently between similar genes, thereby creating a scenario in which recombination is facilitated among gene families sharing significant homology. Recombination of antibiotic resistance gene families creates new allelic variants in which mutations with different evolutionary origins merge, which is the case with TEM and SHV β-lactamases and *qnr* genes (167, 333). Recombination can also produce mosaic genes, merging domains from different gene classes within the same family. Examples include the widespread mosaic genes based on tetracycline resistance, tet(O) and tet(M) (972). Mosaic alleles often present bifunctional activity, such as the aminoglycoside resistance enzymes *AAC(6')/APH(2'')*, *AAC(6')-Ie-APH(2")-Ia*, and *ANT(3'')-Ii/AAC(6')-Iid*, and the β-lactamase, bla$_{LRA-13}$, which is a fusion of a class C (*AmpC*-type) and a class D (*OXA*-type) β-lactamase (973). These fusion proteins expand

the substrate range beyond that of either domain alone, highlighting the important role of recombination in the evolution of antibiotic resistance.

Facilitated gene transfer among relatives: species and clones. The adaptive success of gene transfer depends on the compatibility (relatedness) of the incoming gene (function) and the existing network of functional interactions in the recipient cell, as in physiologically coupled genes (974, 975). Nevertheless, the opposite can also occur if the product of the new incoming gene competes with a functionally relevant orthologous gene present in the genome of the new host and if the fitness costs are high as a consequence of this competition. In this case, gene decontextualization and exaptation can impose a lower fitness cost, allowing the acquisition of resistance genes from nonrelatives. In addition to the integration of the new function (adaptation success) in the new host, structural features are integral to efficient gene transmission. In general, successful gene transfer is more likely to occur between organisms of similar C+G content (less than 5% difference for 86% connected pairs) (976) and/or involving plasmids able to bridge close to distant chromosomal backgrounds (868, 977).

This successful gene transfer would be expected in interactions among relatives, such as among species of Enterobacterales and even in higher taxons as Gammaproteobacteria. All of these organisms are related (with a presumed single common ancestor) and have shared genomic repertoires and congruent evolutionary histories (198).

The eco-evolutionary advantages of relatedness: kin selection. A heterogeneity of phenotypes is expected to occur in time in a sufficiently large bacterial population derived from a single-lineage population, giving rise to a multiplicity of subpopulations that maintains high relatedness but not full identity. This variation allows the global population (a species or quasispepecies) to scan variable adaptive landscapes. The important question here is whether these subpopulations will compete among them or, on

the contrary, whether the members of this community of closely related strains will cooperate to gain common ecological advantages. It has been shown that significant signal interactions (including specific transcriptomic modulation) can occur between closely related strains (978).

The "gain" for the "group of kin populations" expresses the evolutionary weight of indirect selection (those organisms *directly* selected, e.g., because they are resistant to antibiotics) and promotes the indirect selection of kin, genetically-related populations, according to the classic statements by Fisher, Maynard Smith, and Hamilton (979). The "Hamilton rule" indicates that the fitness of the group of kin populations is the sum of those that have been directly and indirectly selected, and the benefit of those indirectly selected is proportional to the relatedness with those directly selected.

Interestingly, the altruist population (the one that has been directly selected, as due to its antibiotic resistance) and the cheater populations might reverse roles over time, a key concept for the "community selection" (as in the case of a species and their clones). The benefit for the altruist-forming part of bet-hedging adaptive strategies (see Section 3.1 Phenotypic variation: bet-hedging adaptive strategies) is that, at a given point in time, one of the cheaters might be directly selected and converted to an altruist and could then indirectly select the old altruist.

EVOLUTIONARY TRAJECTORIES OF ANTIBIOTIC-RESISTANT CLONES AND SPECIES

ARG evolutionary pathways and trajectories and their mobile genetic elements are inserted into the evolutionary events of the bacterial clones and species harboring these genes. What is a bacterial clone? The use of this term is imprecise (980). Does a single

mutation (single nucleotide polymorphism) give rise to a “new clone” or just to a “clonal variant”? As long-term evolution experiments (LTEE) have found (see section Long-term evolution experiments and historical contingency), any ancestor population diversifies, producing an assortment of variants. For the purposes of these studies, these variants are sometimes considered “clones” (981). For the purposes of studying antibiotic resistance, we generally prefer to consider clones as subspecific discrete (distinct) lineages of highly related strains, called clonal complexes, as described in the original multilocus sequence typing (MLST) studies (982, 983) in Bayesian Analysis of Population Structure approaches, which simultaneously consider the frequency of allelic variants and the divergence of groups (984) and the more recent full-sequence phylogenomic studies. These clonal complexes conceptually resemble “ecotypes” that can be defined as sets of strains using approximately the same adaptive space, so that a novel or emergent genotype (mutant or recombinant) outcompetes other strains within such ecotype (985).

A limited number of specialized lineages within bacterial species are frequently amplified under antibiotic selection and greatly contribute to the worldwide spread and transmission of antimicrobial resistance. These lineages are known as “pandemic clones” (986) and “high-risk clonal complexes” (355) among public health and clinical microbiology professionals, respectively. The attribution of an organism to one of these categories allows interventions to be targeted in human and veterinary medicine (e.g., control of hospital outbreaks, infection prevention, vaccination) and risk-assessment analysis to be performed in food safety. Pandemic clones are clonal complexes, fluctuating ensembles of kin clones with periodic emergences of new genotypes. Such variation occurs continuously, assuring a permanent bacterial diversity, so that high-risk clonal complexes are much more stable than a particular clone. Antibiotic exposure is one of the effectors of such diversification, but despite their strong effect in bacterial populations, antibiotics

4079 are newcomers in the field of bacterial evolution. Many spatial-temporal ecological
4080 changes and processes are also involved; consequently, the identification of causal
4081 explanations for the prevalence of a given high-risk bacterial organism is difficult and
4082 does not allow for nomological ("lawful") conclusions.

4083 Despite the apparent persistent (or stable) population structure of *E. coli* in the microbiota
4084 of healthy individuals, clonal expansions of emerging STcs have periodically occurred,
4085 followed by broad diversification. The history of *E. coli* ST131 is a paradigmatic example
4086 of the effects of the trade-off between the natural selection of a clone, intraclonal
4087 diversification, epidemigenicity, and antibiotic resistance. The STc131 of *E. coli*
4088 represents in fact one of the most emblematic examples of an emerging clone reaching
4089 global dissemination (987–989) and can be genetically classified into subclades on the
4090 basis of the serotype, the type I fimbrial adhesion gene (*fim*H), and antibiotic resistance
4091 to fluoroquinolones and third-generation cephalosporins; namely, clade A (*fim*H41),
4092 clade B (*fim*H22) and clade C (*fim*H30), and the H30 subclades C0 (H30, fluoroquinolone
4093 susceptible), C1 (H30-R, fluoroquinolone resistant [FQR]), C2 (H30Rx, FQR+blaCTX-
4094 M-15), and C1-M27 (H30-Rx, FQR+ blaCTX-M-27). The evolutionary history of ST131
4095 clade C isolates is not yet well understood, although a number of studies have hypothesize
4096 the emergence from clade B from an animal origin and a further colonization of clade C
4097 in humans (990). The wide use of fluoroquinolones has led to the acquisition of
4098 fluoroquinolone resistance, which could have contributed to the rapid expansion of the
4099 H30-R clade. The acquisition of F plasmids (990, 991) carrying either virulence or ARGs
4100 conferring resistance to expanded-spectrum cephalosporins (e.g., the blaCTX-M-15 and
4101 blaCTX-M-27 genes) subsequently resulted in a major evolutive advantage that resulted
4102 in the rapid dissemination of the H30-Rx clade (992). Compared with other *E. coli* ExPEC
4103 clones (e.g., ST73 and ST95), ST131 might have developed specialization in the

colonization-infection of elderly patients, a relevant population of hospitalized patients in developed countries, ensuring long-term survival in the nosocomial setting (993).

Evolutionary Dynamics of Resistant clones

The basic theoretical background: Red Queen, stationary, and microbiota-on-a-leash models. The determinants of the diversification and distribution of bacterial genotypes (in our case, antibiotic resistance genotypes) in time and space is a critical issue in the theory of biological evolution. The contribution of population structures and environmental changes to the maintenance of genetic diversity in bacteria has been highly debated in the framework of two major dynamic models: the "Red Queen Hypothesis" (RQH) (994) and the "Stationary Model" (995), respectively. RQH states that populations are structured by biotic interactions, in such a way that one population (or genotype) changes the environment, forcing the others to continue evolving "to keep the place" where they were originally adapted. Initially, RQH implied a constant rate of evolution based on successions of single populations with a common ancestor, as in the classic periodic selection model. Periodic selection purges diversity by the emergence of adaptive genotypes (mutants or recombinants) that outcompete strains within different ecological clusters (ST/clonal complex, ecotypes) (996). Other recent more inclusive models allow progressing in time through coevolutionary oscillations involving several coexisting populations (208, 997, 998). In the stationary model, changes in the population structure are "punctuated" and occur abruptly in response to environmental dis ruptions after relatively long periods of stasis (995, 999). RQH and stationary models are respectively allied to the "gradualism" and "punctuated" end-views of evolution. However, these major evolutionary models are not mutually exclusive, and periods of accelerated evolution coinciding with environmental disruptions can occur. In all of these models, the "ancestor" trunk coexists with the diversified branches, which is suggested

by phylogenetic studies (143). A “killing-the-ancestor” kinetics by more recent lineages, thereby accelerating evolution, cannot therefore be ruled out (1000); in fact, bacterial growth inside colonies is subjected to a similar dynamic (1001).

Finally, when the environment of a local ensemble of bacterial populations is dominated by a hierarchically superior biological entity, and such interaction has been stabilized by protocooperation or symbiosis (e.g., microbiota inside a human or animal host), the evolutionary dynamics of clones and species is also regulated by the host, maintaining microbiota-on-a-leash. That means that the maintenance of resistant species and clones is influenced by variations of the host itself, eventually leading to coevolutionary and co-regulatory processes (1002), finally assuring the functional resilience of the interaction.

AMR occurs in complex and often symbiotic microbial bacterial communities living in dynamic ecosystems in which species and clones are subjected to particular evolutionary dynamics. Hence, the evolutionary trajectories of antibiotic-resistant organisms are inserted into other evolutionary trajectories; for instance, the evolution of AMR organisms inhabiting mammals follows the evolution of the mammals themselves. In a single species (such as humans), the evolutionary trajectories of a particular antibiotic-resistant lineage are determined by the changing ecology of the individual and local group microbiota, the result of conditions such as aging, feeding habits, health status, local environment, hospitalization, drug exposure, and, most importantly, exposure to antimicrobial agents. The resilience of microbiota (inertia to changes) is probably critical in AMR dynamics, and the same population in different species might have different diversification dynamics. The diversity within a population is thus ephemeral, awaiting the next periodic selection event for novel “clearances” (1003). We refer to the term “clearance” because extinctions are rare and vulnerable genotypes can persist as residual

populations, survive different periodic selection rounds, and be “rescued” and further amplified.

Clonal Fluctuations and Evolutionary Rescue

A difficult-to-answer question is does something akin to “the death of the clones” exist, a particularly relevant question in antibiotic resistance (and in vaccination interventions), given that clones are the vehicles of antibiotic resistance. If the clones do not die, they could be fated to diversification. An unresolved issue is whether such diversification is the result of specialization in highly specific niches, which might limit their spread, implying a reduction in population sizes and a higher risk of extinction. There is the possibility of rapidly inverting this risky evolutionary trend by exploiting neighboring niches and compensating specialization with complexity (180) or as a consequence of environmental changes. Such rapid adaptation to avoid extinction is known as evolutionary rescue, a term coined in 1995 with roots in the works by Haldane and Simpson on the evolution timeframe (1004), which become a key concept in the novel eco-evolutionary dynamics field (1005, 1006). The exposure of susceptible clones to antimicrobial agents could hypothetically lead to clonal extinctions. According to theory, the likelihood of clonal populations being rescued depends upon the population size, the supply of genetic variation, and the degree of susceptibility to stressors (1006). The rescue process is influenced by epistasis, HGT (1007), recombination (1008), the cumulative history of stress, the severity and speed of action of antimicrobial agents, general environmental changes, and the population structure, which includes clonal interference (1009, 1010).

However, the most important factor influencing evolutionary rescue under antibiotic exposure likely occurs because of the protection of minority resistant cells to other (neighbor) cells, generally in the same clonal complex, which are spared the biological

cost of producing the resistance trait. The condition is that the resistance mechanism should influence (reduce) the amount of antibiotic in the environment where the susceptible bacteria are placed. The mechanism of resistance, produced by a minority, is therefore converted into a "public good". For instance, a minority of beta-lactamase-producing *E. coli* cells inside a colony are able to protect the whole population, including a majority of antibiotic-susceptible cells, from a beta-lactam antibiotic (1011). Such indirect resistance occurs for most antibiotic-modifying or degrading enzymes, including those acting on macrolides, tetracyclines, and chloramphenicol, but none was detected for aminoglycosides and fosfomycin (1012). These types of collective relations have been examined on the basis of game theory (1013, 1014). In principle, the minority that produces the "common good" resistance should be at a disadvantage, given it concentrates all the costs; the other cells are "cheaters", which have benefits but no costs. This relationship is, however, dependent on the antibiotic concentration, because the resistance mechanism protects the producers more than the neighbor cells. However, the important evolutionary fact is that the resistance trait is frequently located in a transmissible genetic element. By maintaining life in the plasmid-free part of the population, these cells might act as recipients of the beta-lactamase-encoding plasmid, so that the proportion of cheaters will progressively decrease (even more so if the antibiotic concentration rises). At some point, a large number of cells will be producers, and the common good (in large amounts) will then favor the survival of neighboring susceptible bacterial populations. The release of "common goods" (the antibiotic that degrades or inactivates enzymes) favors the survival of the entire population, even if there are no cheaters within. For instance, many antibiotics show an "inoculum effect" such that a dense population tolerates much higher antibiotic concentrations (higher MICs) than diluted or isolated cells (1015, 1016). Thus, the best way to observe the intrinsic activity of drugs is to expose

single cells to various antibiotic concentrations to obtain single-cell MICs, an approach that might help detect the first steps of mutational resistance selection (1017).

The Structure of Clonal Fluctuations

A common observation in studies on the epidemiology of antibiotic resistance is the frequent shifts in the prevalence of bacterial clones, giving the appearance of "oscillatory replacements". The reasons involved in these changing dynamics, the "*structure of the variation*," frequently remains obscure. As an approach to this topic and inspired by the classic concept of periodic selection, Fred Cohan defined types of molecular adaptive changes that determine the frequency of ecological diversity within and between populations (1003).

New hosts' invasion-driven genetic variation. Diversity can be fostered by host invasion, given that variation can increase by adaptations to new hosts. Host-adaptive signatures have been documented in various clonal complexes/sequence types of commensal opportunistic pathogens and frank pathogens responsible for foodborne zoonotic infections (*Salmonella, Campylobacter jejuni*) (1018). Examples include H22 ST131 *E. coli*, which first adapted to poultry and later to humans (990); CC398 *S. aureus* (1019); and CC5 *Enterococcus faecium* (1020). How might a "foreign" invader outcompete (or a least coexist with) well-adapted local strains? One possibility is through genetic variation finding an unexploited niche in the new host that was disregarded due to the success of commensal strains. If fitness is low at the start, the strain can increase in abundance, following something akin to the Sewall Wright metaphor of the shifting balance theory (applied to species) (1021). The possibility of crossing barriers between hosts of resistant clones has major consequences on the evolution of antibiotic resistance. As already mentioned, the bacterial "species" in a single host might be composed of various clones, probably following an oscillatory dynamic (XXX)see later). Transmission

between hosts implies bottlenecks; i.e., frequently only a sample of the clonal composition is transmitted, which favors the spread of particular clones, either stochastically (nonselective bottlenecks) or in a deterministic manner (selective bottlenecks), when the receptor host is suitable to be preferentially colonized by a particular clone (254).

Intraclonal diversification within hosts. There is a variability of niches among hosts and within hosts that drives the variability of antibiotic-resistant clones, which are referred to as "Hutchinsonian niches", imaginary multidimensional spaces in which each dimension represents the variable range of a particular environmental condition or resource required for the optimal growth of a sublineage or particular genotypic group (1022, 1023). Ecological niches are constructed by the hosts and by the bacterial organisms that live there, creating subniches and neoniches that can be exploited by new bacterial genotypic variants. Clonal/strain adaptation to new niches involves strategies of competition and cooperation with other microbes. Within the same lineage, a certain cooperation of adaptive processes, including mutation and recombination, can be expected (1024). Thus, clonal interference is not absolute, allowing for the coexistence of a number of clones with beneficial (adaptive) mutations that might reach relevance with an increase in population size. However, if clonal interference is high, recombination might allow for the maintenance of more beneficial changes in a lower number of clonal entities (1009). In a reduced number of intraspecific clones but with higher cell densities, HGT might have an outstanding facility for favoring the natural selection of adaptive traits, including antibiotic resistance (531, 1025).

Clonalization quashing: genetics and niche variation. Genetic variation allows for the suppression (or near extinction) of an antibiotic-susceptible or antibiotic-resistant clone beyond the possibility of being partially replaced by periodic selection. For

example, a clone that shares most of a single niche with another clone, albeit in different proportions, can be extinguished by an extraordinarily fit adaptive mutation acquired by the second. However, the niches themselves are not necessarily stable over time. This quashing (suppressive) dynamics might occur as a consequence of the variation in niches themselves, by fission, or by fusion with other partially overlapping niches occupying the same spatial regions (1026). In the "emerging new niche", one of the clones that were in partial coexistence might disappear. Such a niche variation is not necessarily global, and clonal quashing would be limited to certain environments. However, if the predominance of a clone occurs locally, the possibility of spreading to neighboring hosts might increase.

Variation fostering cloud or bunch clonal selection. The variation fostering cloud or bunch clonal selection can be considered the opposite of the case presented in the previous section. Adaptive genetic variation might confer an advantage favoring several populations, particularly for kin-clones but also for species sharing the same or neighboring niches (1027), resulting in a "cloud" or "bunch" selection of different bacterial types. HGT is frequently involved in this process; for instance, plasmids serve as vehicles for "common goods", in our case ARGs. Such "bunch" adaptations tend to purge the neutral sequence divergence both within and between populations, while preserving the distinct DNA sequence-similarity of a population/cluster. A poorly explored but interesting possibility is whether the selection of a particular clone leads to a "niche construction process" (1028), which might facilitate the acquisition of kin-related clones, an effect that could have a strong influence on the epidemiology of antibiotic resistance when resistance genes are distributed in different coexisting clones, ensuring the permanence of the resistance trait in a particular patient or environment.

Clonal variation triggering community selection. Local clonal diversification is dependent on the local diversity of Hutchinsonian niches (see above), but such niche

diversity is dependent in turn on the whole structure of the microbial ecosystem, such as microbiota. In a sense, globality is an ensemble of many localities, and bacterial populations should "adapt globally, but act locally" (1027), a concept that suggests the existence of globally adaptive genetic ensembles conferring a selective advantage to all populations constituting a metapopulation, which can result in a "selection of global communities" improving the resilience of the ensemble when confronted with external variation and explains the maintenance of variant clones so that their variation does not jeopardize their ecological links with the community and host. The microbiota has a type of multilevel self-organization. The effects of a single genetic variation in an organism on the entire community embedded in multiple organizational levels is a critical research topic that has recently been addressed with advanced computational methods (1029). Clonal evolutionary trajectories are also determined by this macroenvironment, subjected to external and internal processes, such as trophic (the "intestinal chemosphere") and competitive interactions, leading to multilevel self-organization (141, 1030).

Clonal diversity and antibiotic resistance. How large is the clonal diversity in bacterial species harboring significant ARGs? Most recent diversity analyses have been based on the study of sequence types. In the case of *E. coli*, approximately 10,000 ST-types have been identified in MLST databases. However, in 1992, however, the Orskov´s et al estimated an *E. coli* diversity ranging between 50,000 and 100,000 serotypes (1031, 1032). Taxonomy based on single-nucleotide polymorphisms can be too fine-grained a technique to discern clones. How many *E. coli* clones coexist in a single individual host? Current data suggest that an average of 3.5 genotypes are recovered per host, with some hosts having 6 genotypes (1033, 1034). These data probably underestimate the real clonobiome diversity of *E. coli*. Novel metagenomic techniques to answer this question have just started to emerge. Determining a species' clonal diversity per individual and its

evolution over time is not a trivial task, and determining these phylogenomic aspects are of relevance to understanding the evolution of antibiotic resistance.

Clonal fluctuations and antimicrobial resistance. Long-term analyses of the fluctuations in the prevalence of particular antibiotic-resistant clones in particular human populations are available in certain cases. One of the most emblematic examples of clonal fluctuations is the dynamics of *S. pneumoniae* populations after the implementation of massive immunization programs with pneumococcal conjugate vaccines (PCV) that conferred protection against different serotypes. The wide use of PCV led to a profound reduction in the prevalence of invasive infections and nasopharyngeal carriage of vaccine serotypes among healthy children but produced a compensatory rise in the prevalence of nonvaccine serotypes, commonly referred to as serotype replacement (1035) or serotype switching (1036). Being the targeted PVC targeted serotypes the most prevalent ones on the oro- and nasopharynx, they also collected more frequently antibiotic resistance. Currently, PCV vaccination constitutes the most effective intervention against antibiotic-resistant human bacterial pathogens (1037).

Although the phenomenon of clonal fluctuation in human populations can be better documented in well-adapted species belonging to normal microbiota (such as clonal fluctuation), it is often linked to epidemic events. For instance, clonal shifts in *Salmonella* are highly influenced by events in food safety and food markets and agriculture, including antibiotic policy. Major clonal fluctuations have also been observed in *E. coli* studies, which can be illustrated by changes in the frequency of clones belonging to the major *E. coli* phylogenetic groups, from A and B1 in the 1980s to B2 and F in the 2000s (993, 1038), which illustrates the phenomenon of bunch clonal selection previously mentioned. Clonal fluctuations are also frequent in *Enterococcus* populations (1039).

Most importantly, long-term studies of clonal fluctuations should be differentiated by individual hosts (age ranges are a critical issue), groups of individuals (e.g., particularly the type of hospitalized patient and human communities in different social-environmental conditions), and larger entities (studies in a single hospital, or numerous hospitals, regions, and countries). The study of clonal diversity in each of these groups or compartments should provide different cues for studying the evolution of antibiotic resistance and to establish the resistance wave dynamics.

Traveling clonal waves and antibiotic resistance. Clonal fluctuations resemble wave kinetics and occur at the individual level (inside a single host), in groups and in large host communities, forming landscapes of waves of different amplitudes. In the individual and particularly in open ecosystems (such as mucosal membranes), a bacterial species structure is considered as based on the coexistence of several clones, each one adapted to the situation of a particular spatial-temporal environment, ensuring species resilience; an "optimal clonal composition" of the species. Coexisting clones can be conceived of as alternative stages of the species' population. Due to the fluctuating conditions, certain cells of the best-adapted clone at a given moment will multiply at high growth rates (Figure 8), creating the expansive, leading edge of a pulling wave, which results in increased cell density. The increased bulk of the wave probably contributes to pushing the wave forward, with the result of replacing other clones. The "wave" study applied to the understanding of fluctuations in the spatial spread of biological invasions is a promising field of theoretical research (1040–1042). In the case of antibiotic resistance, the acquisition of resistance in the rising clone might provoke a collapse of other clones; however, the Allee effect in *E. coli* has been shown to frequently impose a compromise between the spread and survival of the species (1043). A poorly explained problem in the dynamics of antibiotic resistance is how the dominant resistant clonal

waves of an individual host influence the invasion of other hosts in the group, producing confluences with similar waves and resulting in larger coupled waves that might increase fluctuations over large distances, as has been detected in other systems (1044). The high geographical propagation velocity of certain high-risk clones suggests the possibility of this "potentiation by coupling waves" hypothesis. Not only might a possible confluence of clonal waves in different hosts promote dissemination, but the rise of a wave in the single host (possibly following the introduction of an external clone) can influence the success of establishing successive kin clones. The first rising population modifies the environment, which can pave the way for establishing the second population (1045). The HGT of adaptive genes (including antibiotic resistance) from the first successes might "convert" other coexisting clones in co-successful resistant clones. Cryptic biological invasions (1046, 1047), either intraspecific or interspecific, trigger rapid range expansion, favoring genetic interactions and the evolution of antibiotic resistance.

Clonal mixtures, range expansion, spatial sorting, and evolution. Multiple initial introductions from genetically distinct source clonal populations at the starting point of the selective process have apparently favored the success and invasion of antibiotic-resistant species (see above "wave potentiation"). That is presumed to happen when these populations are generated or co-occur in expanding populations' spatial edges, which is determined by selection (1048). It has been suggested that these genetic mixtures increase evolutionary potential because of genetic diversity (allelic richness), admixture, and fitness advantages derived from cooperation (see previous section) (1049). Spatial range expansion, the ability of a population or species to disperse and colonize novel areas, is a driver of variability, admixture, and rapid evolution, particularly during the initial stages, with changes in the evolution of cooperation (1050–1052). Traits favoring growth on expanding range edges tends to accumulate locally by this type of "spatial sorting",

generating novel phenotypes (1053). However, mixtures might also produce competition, provoking a persistent “mosaic of maladaptation” in which traits are not distributed in a pattern consistent with adaptation (1054). In any case, if the parameter of “time” is the key dimension in evolution (117), a timeless biology is conceivable, based on the “flow of space” and the resulting consequences for living organisms (1055).

High-Risk Species and High-Risk Clones

The relevant antibiotic resistance threat is “officially” restricted to a few species of 12 bacterial families on the basis of their ability to cause infections and transmit AMR among hosts (WHO, CDC), and a number of them are referred to as ESKAPE microorganisms (1056). However, the “species” should not be considered a significant taxonomic unit in antibiotic resistance. Within these “high -risk” species are genetic lineages almost entirely devoted to antibiotic resistance or that are poorly pathogenic; not all well-adapted subpopulations are equally able to acquire resistance by incorporating exogenous DNA (944, 1057, 1058). Certain other populations infrequently cause infections, with most infections caused by a few well-adapted subpopulations within the species (355). As stated in the previous sections, a “species” can be understood as a complex evolutionary lineage (clonal complex) linked by ecotype-specific periodic selection (985, 1059, 1060). Clonal complexes should therefore be considered as the unit of antimicrobial surveillance. Clonal complexes that are able to increase their abundance by efficient transmission among humans in response to selection by antimicrobials, host immune response, or combined reasons are called high-risk clonal complexes (355) and differ in their population structure, which depends on the inheritance patterns, from highly clonal (*S. aureus, P. aeruginosa*) to highly recombinogenic (*Neisseria* or *H. pylori*), although most opportunistic resistant pathogens lie somewhere between the two (*E. faecium, E. coli, K. pneumoniae*) (1057, 1058).

The available knowledge on the clonal structure of each bacterial species is biased by the overrepresentation in the available databases of strains from human origin, particularly those that are highly pathogenic or antibiotic-resistant. The population structure of the species determines their dynamics as high HGT levels, and recombination highly influences how members of a species diversify, change, and adapt.

Ecogenetics of high-risk species. The bundle clonal structure of most high-risk species determines the resilience of these organisms, multi-adapted and therefore following the principle "never put all your eggs in one basket" principle (61). In many of these cases, the resulting ecological diversification is highly dependent on a large "accessory genome"; i.e., from genes that are found only in different fractions of the species' global population. Such ecological diversification ensures greater possibilities of contact with other species, potential donors of new adaptive genes (including antibiotic resistance), which enlarges the accessory genome. However, the alternative strategy is also effective: high niche specialization, particularly in small niches or subniches, can be achieved using a larger "core genome" with specific local variants, as occurs in *Listeria monocytogenes* and *Legionella pneumophila.* However, "small niches" or bacteria exclusively adapted to specific niches reduce the possibility of interaction with other species that might act as donors of adaptive genes, which might explain why these organisms are less successful in acquiring antibiotic resistance (545).

EVOLUTIONARY TRAJECTORIES OF ANTIBIOTIC-RESISTANT COMMUNITIES

Microbial communities (or microbiomes) are also evolutionary individuals when they are interactively associated with a particular environment and act as other entities of lower

range in the biological hierarchy. As with other units of evolution, microbial communities evolve by trade-offs between dispersal and colonization, related to *r*-type and *K*-type strategies (1061).

We understand here as antibiotic-resistant communities those microbiotic ensembles that have been modified in its composition due to the effect of antibiotic exposure and antibiotic resistance. Short-term, transient modification of these communities promoted by antibiotics are frequently reversible. If resistant organisms become prevalent, however, they might cause long-term, or even permanent changes in microbial communities. In fact, this is one of the more severe global threats related to antibiotic resistance, not necessarily linked in this case with human health but with the global health of the biosphere.

The trajectories of Microbial Communities

The interactive network among biological entities that are components of microbial communities is subject to evolution. The building up and the homeostasis of microbial communities implies group interactions, which generally cannot be reduced to the corresponding addition of pairwise interactions. A modular organization of communities into subsystems constituted by groups of species contributes to their stability (1062). Their modular ecological structure has probably evolved due to the cost of maintaining network interactions (1063). Variation and evolution within one species can shape the ecological properties of entire communities; in turn, the community context can govern evolutionary processes and patterns. Therefore, we need a convergence between research in community ecology and in organismal evolutionary biology (1064). Ecological interactions, leading to within-population variation and ecological specialization (1065, 1066), are a source of selection that can drive local adaptation and speciation. Conversely, the evolution of these populations in response to such selection can result in a feedback

4451 that modifies species interactions, communities, and ecological dynamics. First, genetic
4452 variation affects communities; second, multispecies interactions cause diffuse selection
4453 and geographic mosaics of selection; third, there are macro-evolutionary consequences
4454 of multispecies interactions (1067).

4455 We cannot rule out that antibiotic use, which alters community networking, might
4456 promote modularization and hence the possibility of the inter-host exchange of species
4457 groups. The intensity and maintenance of these changes is proportional to the duration of
4458 the antibiotic exposure, at least in the first stages of the process. In addition to its
4459 importance for human health, this relationship is one of the main reasons behind the need
4460 for reducing extensive antibiotic use in humans and animals; particularly, the release of
4461 antibiotics into the environment and the prevention of deleterious changes in the structure
4462 of normal microbial communities.

4463 Antibiotic exposure alters the proportion of species within bacterial communities. Most
4464 antibiotic resistances, including those acquired by HGT, occur in the minority populations
4465 of microbiota, which can rise to “abnormal proportions” within their communities. As
4466 previously stated, this increase in relative population density frequently leads to clonal
4467 diversification, contributing to a more effective exploitation of the environment and an
4468 improved and more permanent adaptation of these clones to the environment
4469 (phylogenetic clustering). The net result is that, once these “better adapted clones”
4470 emerge, they can be maintained even *in the absence* of antibiotic exposure. Better
4471 exploitation of the host’s resources generally implies facilitated transmission between
4472 hosts, particularly in highly fragmented pathosystems with low connectivity (1068).

4473 The change in proportions of certain focal taxa (the resistant ones) exert an “ecological
4474 pressure” on the rest of the community, should be “reshaped” in composition to assure
4475 the maintenance of the whole microbial consortium and its optimal equilibrium with the

environment. To a certain extent, the community should co-evolve with the resistant taxa. Such evolution is probably a sequential process caused by reciprocal natural selection between species (diffuse coevolution), which can vary from one resistant species to another, depending on their location (betweenness centrality) in the community network. The rate of community variation explained by the variation in particular resistant species (community heritability) starts to be understood by the use of deep metagenomic techniques.

Resistance at the microbial community level. When a given microbiotic ensemble interacts permanently with a particular environment, such as human and animal intestinal microbial communities, a positive interaction is expected between microbes within the community and with the host. In most cases, this interaction was established millennia ago and expresses a coevolutionary relationship. In terms of the microbial community, this interaction can be expressed as niche conservatism, the tendency for bacterial species to retain ancestral traits that ensure the species' original (selected, historical) functions within the microbial consortium (1064). To maintain such homeostatic behavior in open environments, bacterial organisms might have evolved traits that protect their interactive network (resilience traits), including those affecting antibiotic resistance. Bacteria endowed with resilience traits do not need to evolve by acquiring resistance genes, which might influence their fitness and their interactive network within the community. Resilience in fact opposes the evolution of resistant microbiotas.

In an antibiotic-polluted world, a number of bacterial species have recruited specific resistance mechanisms carried by MGEs. These MGEs frequently correspond to populations that have been selected in the past by antibiotic exposure, in the same host or in a connectable host. In a certain sense, the MGE population in the community keeps a historical record of previous selective events. In case of re-exposure, the MGE population

employs a strategy similar to immunological memory in B cells and T cells in vertebrates; the “mechanism of resistance” is distributed by HGT among susceptible relatives. This strategy spares the need for harboring resistance genes, imposing certain fitness costs (including the cost of MGEs) on the host cell, which partly explains the fact that certain susceptible bacteria survive and that the curve of resistance prevalence in most susceptible species levels off at a certain proportion. Thus, resistant bacteria protect the susceptible ones by providing genes and detoxifying the local antibiotic. There are a number of theoretical studies (game theory) on cooperator (resistant altruists) and cheater (susceptible) members of a microbial group (381, 1069–1072). If lateral gene transfer specifically protects phylogenetically close populations, detoxification protects the entire community (at least the spatially related, “granular” community).

For example, a small proportion of TEM-1 beta-lactamase *E.coli* cells can protect an entire susceptible colony from ampicillin (1011), which should also occur in cases of protection of other susceptible bacterial species by resistant ones. The dense anaerobic populations of the gut can provide beta-lactamases able to significantly degrade penicillins, cephalosporins, and carbapenems (1073, 1074), ensuring the maintenance of susceptible organisms. It has been shown that antibiotic selection for particular resistance traits in a given organism also occurs in the context of a complex microbiota; however, selection appears to be limited by the possible degradation of the selective agent (1075) or increases the cost of resistance (1076). These cooperative ecological effects occur and evolve for many traits other than antibiotic resistance (typically for colonization and nutrition) in complex, patchy microbial communities (173) such as the intestine (1077). In fact, antibiotic inactivation by degradation can be followed by further enzymatic degradation of the antibiotic’ carbon backbone, taking nutritional and energetic advantage

of the former antibiotic, for the degrading bacteria and the surrounding community (1078, 1079).

Evolutionary trajectories in human microbial communities. The evolution of complex systems such as integrated microbial communities is slow compared with that of discrete populations, given that the interactive network provokes a high degree of robustness. Robustness and evolvability are two opposite trends in natural complex systems. The combination of *ex unibus plurum* (diversification, evolution) and *ex pluribus unum* (unification, robustness) processes (180) ensures the plasticity of microbial communities. A key point is the understanding that many microbial communities (such as the intestinal microbiota) should be reassembled from its components with high frequency (as in all sterile newborns). However, the composition of the final ensemble (a functional ensemble, with species that might vary but provides the same function) is remarkably constant for a given type of host, to the extent that the community *replicates* as a biological unit (124). The construction of the microbiota can occur following complex interactive codes integrating mutualism, as well as competition (1080) among the members of the community. The trajectories required to achieve the final integrated pattern might originate from different members pioneering the colonization process (1081) and establishing niche segregation colonization patterns (1082). Therefore, not all of the pieces in this puzzle have identical sizes, creating inter-host differences based on the dominance of certain pieces that determine "puzzle regions". The existence of community composition types (enterotypes) illustrates these differences (1083). In any case, there is a remarkable stability in the organisms hosted by a particular individual, suggesting a constancy in the individual patterns of antibiotic resistance and antibiotic resilience (1084). The initial microbiome composition (including enterotypes) determines its reshaping by antibiotics (1085).

Patients intensively treated with antibiotics over decades are a source of resistant bacterial populations enriched in number by selection and consequently by host-to-host transmission. These resistant organisms overflow the patient's bacterial compartment to integrate "the normal microbiota" of healthy, nontreated individuals. Transmission of resistant organisms can occur from mothers to newborns, from treated patients to relatives (1086), and in travelers exposed to other microbiota (1087).

Changes in human behavior and demography might contribute to the fixing of human-related antibiotic-resistant communities. As significant antibiotic resistance becomes concentrated in certain populations of Proteobacteria and Firmicutes, conditions promoting their proportional increase in the intestine will augment antibiotic resistance. These conditions include malnutrition (particularly in children and frequently associated with intestinal overgrowth) (1088), a high-fat diet, obesity (1089), older age (1039, 1090), and travel to areas with poor sanitation (1087).

If, in the long term, resistant Proteobacteria and Firmicutes are consistently increased as components of the human microbiota, the entire microbial community is expected to evolve to explore novel equilibrium possibilities. In a complex system, global re-adaptations following significant local changes are expected. The evolutionary and clinical consequences of such modifications (new equilibria) remain to be explored. We cannot rule out the possibility that the community evolution of antibiotic resistance might reach evolutionary stasis, either because antimicrobial agents are no longer required for treating infections (imagine a new era based on controlling the host response to bacterial challenges) or simply by the *erosion of resistance fitness peaks*. As stated before, once resistance and resilience reach a certain level in normal microbiota, the selective effect of antibiotics should decrease. For a number of drugs, the previous selection of antibiotic-resistant populations with drug-inactivating mechanisms produces a massive degradation

of the antibiotic, resulting (in the case of challenging communities) in antibiotic selection not necessarily acting in a dose-dependent manner (1075).

The antibiotic-induced alterations of the microbiome might have consequences for host health. The critical issue is the abnormal increase in absolute population size of potentially pathogenic minority populations, presenting substantial resilience to antibiotics and improved capacity to acquire resistance, as is the case for humans with *E. coli*, *E. faecalis*, and *E. faecium*, bacteria that rank the highest for bacteremic and urinary tract infections. Depending on their number, these organisms migrate into the urine and translocate across the intestinal wall (frequently stochastic translocation). The number of bacteremic episodes therefore increases, particularly in debilitated hosts (1091).

Evolutionary trajectories and microbiota community coalescence. Over the last century (although the process started in the Neolithic period), communication among environmental, animal, and human microbiota has been greatly facilitated by anthropogenic intervention as a consequence of increased environmental overlapping, the world homogenizing power of globalization, and the asymmetrical increase in the number of individuals in the planet's various biological species. Along with the increase in human population size, there has been a simultaneous increase in the population size of highly uniform food animals. For instance, the cattle inventory in 2018 was one billion head, and half of the world's stock of approximately 23 billion chickens are highly genetically homogeneous (by artificial selection of the most productive breeds) and are fed identically, thereby producing parallel increases in the microbial populations contained in their microbiota, consequently enhancing the possibility of merging human and animal microbiota, known in ecology as "community coalescence" (1092). The combined increase in the number and the reduction in diversity of animals interacting with humans should facilitate reiterative coalescence events between their microbiotic populations, a

type of merging microbiome and hybridization that might give rise to (at least partially) novel assemblies of bacteria. Given that antibiotic-resistant bacteria originating in a particular microbiome are frequently de-adapted to be efficiently inserted in others, shared microbiomes should facilitate the spread of antibiotic resistance (of human, animal, or environmental origin). Particularly important in the dissemination of resistance at the community level are shuttle bacterial groups of generalist species (or clones within species) able to multiply in the microbiomes of various hosts, including humans, animals, and plants (514).

Coalescent microbiota (the degree of coalescence will need to be measured in more detail in future research) also encompass free, natural environments. One consequence of gut colonization is the net increase in the density of resistant populations that are excreted into the environment. Resistome composition across habitats is generally structured by bacterial phylogeny along ecological gradients, with strong interactions between human populations and polluted environments, particularly in low-income habitats with poor excreta management strategies (1093). A continuous flow of resistance genes from polluted environments, which contaminates water supplies and food uptake, ensures the growing integration of resistant organisms into normal microbiota, which is favored by the use of antimicrobial agents, resulting in the human intestinal resistome becoming enriched in populations from high-consumption countries (1094). The number and variety of possible antibiotic resistance trajectories (from genes to communities) should be increased by microbiota coalescence and by the interchange with environmental populations.

Antimicrobials Influencing Ecology and Antibiotic Resistance in the Environment

Ecological and evolutionary processes frequently operate on similar timescales (1095). With the exception of resistance acquisition in pathogens by recombination with genes

originating in commensal organisms sharing the same microbiota, the primary event of novel resistance acquisition is not expected to occur in clinical settings but in ecosystems where the environmental donor and the pathogenic receptor meet (1096).

The importance of anthropogenic antibiotic pollution in the environment is based on the selection of low-level, frequently unspecific mechanisms of resistance in a very large and heterogeneous ensemble of bacterial populations. The antibiotic-polluted environment acts as a "training school of resistance" for physiological mechanisms that might lead to efficient resistance traits across microevolutionary events. Antibiotic resistomes, including MGEs, are significantly enlarged in peri-urban areas (1097, 1098).

There is an expected correlation between biological abundance/diversity and environmental diversity, and biological hierarchical structures should correlate with niche hierarchies (1099). Considering that microbial environments are highly complex and structured, some of their components might progress to higher fitness (resistance) peaks. In the presence of antibiotics or other pollution these resistant organisms might increase in number, facilitating further variability and evolution of resistance traits (1100). Better tools are urgently needed for establishing the selective forces acting at microenvironmental (submillimeter) scales (1100, 1101). Next-generation sequencing technologies will be pivotal in this endeavor (1102).

At macroenvironmental scales (such as soil), microbial community-level evolutionary processes leading to long-term modifications have been poorly studied. The changes are probably shaped by a mixture of deterministic forces, pushing communities to their specific niches, and frequent neutral, stochastic events (1103). In natural environments, antibiotic-resistant populations and the communities hosting them are in close contact and interact with many other biological entities, such that changes in the biosphere and

microbiosphere should have consequences in the distribution of antibiotic-resistant populations (10).

Selection of Antibiotic Resistance by Nonchemotherapeutic Antimicrobial Inhibitors

Biocide compounds, including disinfectants, antiseptics, heavy metals, food preservatives, and detergents have been increasingly employed to reduce bacterial contamination. How the considerable biocide exposure of the bacterial world influences the evolution of antibiotic resistance is a matter of concern. However, acquired, inheritable resistance to biocides remains rare (18), and the selection of antibiotic resistance by biocides is infrequent. Interestingly, numerous biocide-resistant mutants have shown increased susceptibility to certain antibiotic compounds, which specifically act on cell envelopes such as the cell wall (beta-lactams) and cell membrane (poly-L-lysine, polymyxin B, colistin, antimicrobial peptides). Biocide-resistant mutations (single-nucleotide polymorphisms) are frequently found in genes that have a role in energy production, membrane biosynthesis amino acids, and transport (1104, 1105). As it is known, there is a strong connection between cell-wall and membrane growth, determining the frequency of cell division (1106).

Physical disinfection with ultraviolet irradiation is employed in water treatment plants. Ultraviolet-light-emitting diodes are a useful tool for reducing bacterial loads without releasing disinfectant byproducts; however, it requires appropriate disposal facilities to prevent mercury release, potentially affecting the selection of metal-antibiotic-resistant bacteria.

Among chemical disinfectants, chlorine is classically the most commonly employed antimicrobial, acting on bacterial DNA and producing membrane-lethal alterations. A number of authors have suggested that certain *E. coli* strains have a better ability to survive in sewage treatment plants that employ chlorination and UV irradiation for disinfection (1107) and that tetracycline-resistant strains in particular might be less decontaminated by treated water (1108). However, there is likely no strictly acquired/inherited chlorine resistance in bacteria. A number of cells can be more resistant to chlorine decontamination, but this is essentially due to phenotypic resistance/tolerance (mostly due to adhesion-aggregation to particles) and biofilm formation with the production of extracellular protective polymers. These effects might be triggered by sublethal chlorine concentrations, which might transiently increase the expression of antibiotic resistance (1109). In general, however, chlorination and other alternative strategies (such as peracetic acid preparations (1110) appear to have low (if any) effects on the evolution of antibiotic-resistant organisms.

Selection of Antibiotic Resistance by Water Decontamination Procedures

Urban wastewater treatment plants might be considered as one of the hotspots in the release of antibiotic resistance into the environment (136, 1111). A number of nonantimicrobial procedures have been classically applied to wastewater treatment plants, with variable effects on decontamination of antibiotic-resistant communities (1112). The application of membrane bioreactors, sequencing batch reactors, and activated sludge has significantly reduced the density of resistant populations in water, in contrast with biological filtering and upflow anaerobic sludge blanket technology (1113). When anaerobic sequencing batch reactors were employed to treat pharmaceutical wastewater containing sulfamethoxazole, tetracycline, and erythromycin, multiresistant

organisms were detected in the reactor's effluent (1114); however, enriched ARGs frequently belong to nonpathogenic bacteria (1115).

Sewage treatment plants exert a powerful modifying force on the species composition of the incoming contaminated water, which influences the amount and type of resistance genes, making the selective effects of antimicrobials in the effluent difficult to assess (1116). However, meta-analyses have shown that composting and drying significantly reduce the relative abundance of resistance genes and MGEs in organic waste but only marginally in anaerobic digestion (1117).

The selection and evolution of antibiotic resistance in soils is likely enhanced by common fertilization strategies (e.g., nitrogen fertilizers strongly affect the soil content of ARGs) (51). It s difficult to imagine decontamination procedures, which might have deleterious ecological effects.

The Interplay of Antibiotic Resistance and Virulence

We previously and extensively addressed the interplay of antibiotic resistance and virulence in a review in this journal (352). Antibiotic resistance, virulence, transmission, and general bacterial fitness are closely linked processes, with a high degree of cross-epistasis and coevolution of the involved networks. Methods have recently been proposed to investigate such interactions from a systems biology perspective (1118). However, the definition of "virulence genes" and "pathogenicity genes" remains extremely confusing. The likely reason is that to be pathogenic, the organism should be endowed with traits facilitating establishment in the host, and most so-called "virulence genes" encode for colonization factors. Paradoxically, organisms less adapted to colonization might be more pathogenic, pushed to invade empty spaces out of the highly competitive areas where the normal microbiota is located. Given the long-term adaptation between hosts and

4720 microbiota, the most abundant bacteria in human or animal hosts are rarely the more
4721 virulent ones. Efficient colonizers have higher cell densities and wider access to genetic
4722 interactions, favoring the acquisition of antibiotic resistance. For instance, the more
4723 resistant populations (e.g., serotypes) in *S. pneumoniae* are the more abundant but not the
4724 more pathogenic ones.

4725 A number of examples in which antibiotic resistance is associated with lesser virulence
4726 are presented below. The constitutive hyperproduction of chromosomal AmpC beta-
4727 lactamase reduces bacterial fitness and virulence. Vancomycin-resistant *Enterococcus*,
4728 colistin-resistant *Acinetobacter* strains, and porin-deficient carbapenem-resistant *P.*
4729 *aeruginosa* are less virulent in animal models and frequently in the clinical setting (1119,
4730 1120). Multidrug-resistant mutants of *P. aeruginosa* involving efflux pumps are also less
4731 virulent (1121). In neonatal sepsis caused by *bla*NDM-1-positive Enterobacteriaceae,
4732 mortality was lower (13.3%) than for cases caused by *bla*NDM-1-negative (22.2%)
4733 (1122). Fluoroquinolone-resistant *E. coli* tends to have fewer virulence factors than
4734 susceptible ones (1123) and are less pathogenic (1124). In *Staphylococcus aureus*, there
4735 are no differences in clinical virulence between MRSA and MSSA, and mupirocin-
4736 resistance acts epistatically, reducing pathogenicity traits (626, 1125).

4737 Epidemics caused by multiple antibiotic-resistant clones ("high-risk clones") are however
4738 a major cause of morbidity and mortality, constituting a recognized worldwide public
4739 health problem, which appears to contradict the statements of the former paragraph. The
4740 main reason for this apparent paradox is that the selection of antibiotic-resistant
4741 populations through the use and release of antimicrobials increases the absolute density
4742 of resistant cells (1126). By reducing the fitness of competitors, antibiotics act as a
4743 "colonization helper" of resistant populations. The outcome is an increased frequency of
4744 resistant populations, resulting in a number of consequences. First, the high frequency of

resistant cells increases the ability for host-to-host transmission, particularly in hospitals and farms, with a high level of antibiotic exposure. Second, the increased frequency favors the access of resistant cells to other bacterial populations, which are potential donors of new antibiotic-resistance genes and virulence-colonization determinants. Third (and most importantly), the absolute density of resistant organisms in the gut increases the likelihood of invasion of the host's tissues. Translocation from the gut to submucosal spaces and the bloodstream (sometimes producing bacteremia) and spread by contiguity with the urinary tract are essentially stochastic events that are proportional to cell density; if resistant populations prevail, the risk of bacteremia by such organisms increases (1127). Lastly, as invasive infections caused by resistant organisms increase, a larger number of novel antibiotics are employed, alone or in combination, favoring the evolution toward multiresistance.

PREDICTING EVOLUTIONARY TRAJECTORIES

Paraphrasing Lobkovsky, Wolf, and Koonin, (1128), the predictability of evolution depends on our knowledge of the fraction of the trajectories in fitness landscapes that are accessible for evolutionary exploration. In other words, predictability depends on our knowledge of the evolutionary constraints influencing (in this case) the development of antibiotic resistance (1129). Several types of explorations are possible; however, they are currently insufficient for going beyond the quest for general principles and providing solid predictions. A frequently employed test for predicting mutational paths is the repeatability of evolutionary trajectories in replicate populations, which depends on the emergence rates of variants, their fitness effects, and their interactions (including epistasis). These experiments might be complemented by directed research, constructing site-specific mutagenesis genotypes with all single and combined mutations that are predicted to be under positive selection in phylogenetic analysis or evolution experiments

and subjecting them to controlled selective environments (181). These empirical fitness landscapes (1130) include exposure to different antibiotic concentrations and combinations or sequences of antimicrobials and changing nutritional or growth conditions. Experimental evolution methods, using the serial passage of bacteria in culture media with selection at constant or varying concentrations of antibiotics, have been employed to determine mutational trajectories (181, 1131).

Experimental Evolutionary Trajectories

Long-term evolution experiments and historical contingency. Experimental evolution is the study of evolutionary processes occurring in experimental populations in response to conditions imposed by the experimenter (618). The hallmark of the studies on experimental evolution in microbiology is the famous Long-Term Evolution Experiment (LTEE), launched in early 1988 by Richard Lenski to test the repeatability of evolutionary dynamics across replicate populations (1132–1134). In LTEE, 12 replicate *E. coli* populations were placed into tubes of minimal liquid medium containing glucose as the limiting resource. In this "from here to the eternity" experiment, 1% of each culture was seeded into fresh media every day. Every 500 generations, the remainder of each population was frozen to keep a record of the accumulated changes for further studies (1135). Currently, evolution to 70,000 generations in what appears to be a stable scenario (only population fluctuations in seeding a new tube each day) has been achieved. The study highlighted that evolution, even in a simple scenario, is an intriguing mix of random (mutation and drift) and directional (natural selection) processes. The generation of mutants might produce negative genetic interactions offering a possibility for new beneficial mutations to emerge (615). In general, these studies show the never-ending history of bacterial evolution: after so many generations in a constant environment, there

are sustained fitness gains in variability, implying that both adaptation and divergence can continue, possibly indefinitely (?) (1136); a lesson that applies to antibiotic resistance. The Shakespearian question to apply here is whether “evolutionary time will reach an end” (entropic evolution).

Parallel evolution can be shown in LTEE. The fitness of *E. coli* in extracting all possibilities from the culture medium (and the products released by bacterial metabolism) increased during the experiment, and the trajectory of changes explaining these gains was similar across the replicate populations but not identical. Mutations were consistently fixed in a number of genes in all 12 populations (1137, 1138), although the exact mutations at the sequence level differ in almost every case. A parallel evolution among replicates in gene expression was also demonstrated, due to parallel changes in a gene encoding a “global” regulon. However, divergent evolution was also detectable. For instance, the emergence of the ability to use citrate occurred in only one of the replicates; some of the lines evolved the inability to use maltose.

The citrate-using variant only emerged after 31,500 generations, as a random, fortuitous historical contingency. The expected frequency for such a variant is less than $3x10^{13}$ per cell and generation (1139). A genetic prehistory of the ability to use citrate was detected in three coexisting clades (within the same replicate) that evolved a tandem duplication, increasing the expression of a previously silent citrate transporter (1140, 1141). However, only one of the three clades developed a significant citrate-using phenotype, indicating the need for further changes allowing expression. The term “potentiator genes” was coined to refer to the genes involved in the prehistory of the citrate-using phenotype (because they were considered to potentiate the emergence of such a phenotype); however, this is a misleading term. The cryptic variation might depend on the presence

of buffering mechanisms, and the phenotypic expression might derive from the release of such mechanisms.

Historical contingencies are expected in antibiotic resistance trajectories. Bacterial populations, with huge population sizes, are spread in a vast variety of changing environments, and therefore the accumulated "time-history" of bacterial lineages is extremely high (117). As in the case of citrate utilization in LTEE, silent mutations might arise by historical contingencies that eventually facilitate the emergence of significant antibiotic resistance. These accidents will determine or produce the extinction of particular evolutionary trajectories. Given the random nature of most environmental changes, however, trajectories, at least in these initial stages, will remain largely sensitive to history and are therefore unpredictable (1141), thus precluding the "replaying of the tape" of evolution (1142). Even in simple experiments such as LTEE, there are obligatory bottlenecks in transferring inocula from a culture to a new flask with sterile broth or inadvertent subtle changes in experimental conditions that might produce unexpected rare chance events leading to unexpected results, a situation defining accurately "historical contingencies" (1143). Of course, nature differs from test LTEE identical tubes; in the case of antibiotic resistance (and many others), microorganisms with identical ancestor are be placed in multiple environmental circumstances but submitted to the same selective force; previous adaptative events dictate the trajectories of later evolutionary processes (1144). Because of that, clones or species might have different evolutionary trajectories (1145).

Evolution in empirical fitness landscapes. The desirable combination of LTEE and realistic empirical fitness landscapes is still far from our technical capabilities, given that this combination implies "sequential replication of landscapes". However, the approach provided by the Kishony's group (1146) in which bacteria spread and evolve against a

large antibiotic gradient in soft agar mega-plates is promising in this respect. Empirical fitness landscape studies are based on the artificial mix of various mutations that presumably influence fitness (such as antibiotic resistance) and on studying the fitness of these genotypes and their epistatic combinations (1147). The main problem is sampling to detect all variants present in the local population, across the gradient, with which to perform independent fitness studies. However, a reasonably good resolution of these fitness landscapes might be obtained in the analysis of areas exposed to strong selection (1148).

A turn in modeling complexity results from the need to merge different selective fitness landscapes, changing the selective environments. The goal is to measure the organism's fitness in a new environment, which had different fitness in a different environment. This approach might be achieved by "two-step" evolution experiments, starting with an LTEE in a particular environment. The subsequent replicate populations are then transferred and propagated for a new LTEE in a new environment (1149). There is a clear interest in the evolution of antibiotic resistance (e.g., for detecting changes leading to multiresistance and antagonistic pleiotropy in particular variants to different antibiotics). Environmental changes might produce evolutionary constraints and tradeoffs (correlated changes move in opposite adaptive directions), which might create conflicts between the survival and reproduction components of fitness (211).

Advances have been made during the past decade in constructing various types of miniaturized, automated fitness monitoring applications for *in-vitro* evolution (i.e., evolution machines). Particularly promising are the applications that take advantage of microfluidic technology, creating "microfluidic landscapes" (1150). Combined with live-cell imaging, microfluidics can help address the issues regarding the relationships of

physiological phenotypical adaptation, selection, and inheritable resistance (1151) across fitness landscapes.

Directed experimental evolution of antibiotic resistance. Directed experimental evolution experiments are those that are designed to evaluate the possibility of obtaining successive best-fit antibiotic-resistant variants under controlled exposure to antibiotics. These experiments are frequently based on serial passages of a culture containing the ancestor(s) population, thus ensuring bottlenecks in the daily propagation of a sample from one culture tube to the next. The successive culture tubes typically contain growing antibiotic concentrations, and the objective is to obtain the variant with the highest MIC and to explore the mutational path that has produced such a variant (648). On other occasions, the goal of experimental evolution is to ascertain the possibility of obtaining variants that broaden the spectrum of antibiotic inactivation. As previously stated, an efficient method is to identify (by genetic analysis) altered positions in the sequence of the resistance gene that have likely been submitted to antibiotic positive selection, to construct these mutants and their combinations by site-directed mutagenesis, and to sequentially expose the corresponding cultures to growing concentrations of the various antibiotics. These studies frequently reveal the possibility of diversification into several possible pathways (181, 473). Direct genetic reconstruction of available trajectories might consider not only antibiotic resistance but also compensatory evolution and enzyme stability (599).

Experimental evolution of fitness costs of mutational variation or acquisition of resistance genes. Antibiotic resistance usually comes at a fitness cost, due to the fact that resistance mutations typically target important biological processes in the cell (598), whereas the acquisition of resistance via HGT is typically associated with the costs imposed by MGEs (749). Quantifying fitness costs and their reversibility is critical to

predicting the resistance determinants most likely to succeed and to affect evolutionary trajectories. Several techniques are available to measure fitness costs. The simplest method is to measure the growth rates of resistant and ancestral clones by monitoring optical density during growth as monocultures (often employing multi-well adapted spectrophotometers). Growth rates can be employed as a proxy for fitness; however, this method is not overly sensitive to small differences in fitness. More accurate estimates of fitness can be obtained by performing pairwise competition experiments, which measure the change in the ratio of two strains after growth as mixed cultures (1152). Competition experiments are preferred over single-culture techniques because they integrate several growth parameters, such as lag phase, growth rates, and efficiency of resource usage (1153). New computational methods have been proposed to predict the outcome of competition experiments from growth curve data (1154). Competition experiments can be performed in test tubes or *in vivo* by infecting model animals with a mixed bacterial culture, which is likely to provide a more realistic view of the competitive ability of antibiotic resistance mutants.

However, the cost of antibiotic resistance is itself an evolvable trait. Bacteria readily acquire secondary mutations that alleviate the fitness costs associated with resistance. This process, known as compensatory evolution, can be reproduced under laboratory conditions. Most experimental designs consist of propagating resistant bacterial clones during a relatively large number of generations while frequently monitoring for fitness gains using the above-described methods (1155). Propagation is typically performed by cycles of dilution and growth (serial passages) of the selected bacteria, either in the presence of the antibiotic to which the test clones are resistant or in antibiotic-free media. Alternatively, continuous growth of the microorganisms can be achieved by the use of chemostats and bioreactors, in which a constant supply of nutrients is provided to allow

the microorganisms to grow steadily (1156). Although *in vitro* compensatory evolution experiments have provided invaluable insights into the evolution of antibiotic resistance, evolutionary trajectories crucially depend on the environment. Compensatory evolution experiments performed *in vivo* often lead to different results than those from experiments performed with test tubes (580).

Directionality and Repeatability of Evolutionary Trajectories

Based on the notion of contingency (the impossibility of determining whether something is either true or false under every possible evaluation) and using primitive computer modeling, the paleontologist Stephen Jay Gould expressed in 1990 his deep concerns about the repeatability of evolutionary trajectories and outcomes by "replaying the tape of life". (1157). Since then, the contingency-convergency debate has remained central in evolutionary biology. Noncontingency occurs (e.g., in developmental genetics), and conserving a complex solution to an adaptive problem is frequently simpler than repeatedly reinventing the solution (the "if it ain't broke, don't fix it" maxim) (1158), which applies to evolutionary trajectories in antimicrobial resistance, at least for bacterial organisms of the same lineage (the "inventor"). It is possible that different evolutionary trajectories might recruit variant steps or insert functionally equivalent changes without altering the final phenotype or even that different trajectories produce an identical result (convergence). The reality of convergent evolution (independent origin of similar functions) suggests that iterated evolutionary outcomes might be identified because they follow a seemingly law-regulated determination (1159), in contrast to Gould's thought experiment. However, numerous studies have revealed the surprising result that developmental pathways do in fact diverge throughout time, even with no accompanying change in the phenotypic outcome. Very close trajectories at the start of the process are expected to rapidly diverge, given that divergence is exponential (1160).

Pathways and trajectories can, however, be limited by possible changes in adaptive proteins (654). The increase in population size might have a reduction effect in *E. coli* fitness trajectories (271), but this effect might be nonmonotonic, depending on the supply of beneficial mutations (1161).

Whole genome sequencing has recently been employed to study the reproducibility of adaptive trajectories. In general, adaptive convergence explains the increased reproducibility of the advanced steps in the trajectory once a favorable phenotype (not necessarily a fixed constellation of mutations) is obtained (1162). Assessment of potentially advantageous phenotypes can be obtained by experimental fitness assays, including the study of substrate-binding affinities of mutant proteins (1163). Results from genetic reconstruction experiments indicate the predictability of the associations between antibiotic-resistance chromosomal mutations and fitness and suggests that epistatic effects are rare even when up to four mutations are combined (623).

Complex Parametric Space, Chaotic Trajectories, and the Strange Attractor

The predictability of evolutionary trajectories of antibiotic resistance depends on a number of factors (constraints): the rate of resistance trait acquisition; the resistance phenotype; the fitness of the resistant organisms as a function of drug concentration; determining the strength of selective pressures; epistatic interactions and compensatory evolution; co-selection of other resistances; population bottlenecks; and bacterial interactions (1164). A more detailed list of parameters (quantitatively) for establishing the conditions that might shape evolutionary trajectories has recently been proposed (41).

The ensemble of these parameters (to add complexity, frequently in the form of composite parameters) constitutes the parametric space, composed of six basic parameter ontologies: 1) *contact rates,* the probability that two particular evolutionary units could be in close contact during a sufficiently long period, enabling potential interactions; 2) *transmission rates*, the probability that one evolutionary unit moves into another unit of the same or different hierarchical level; 3) *integration rates*, the probability that one transferred unit could be stably maintained in coexistence with another unit or assembled with other units; 4) *replication rates,* the probability that a particular unit will increase in copy number at a certain speed and reach certain final densities; 5) *diversification rates,* the probability that a particular unit produces genetic variant units at certain rates and variants of these variants; and 6) *selection rates,* the probability that a particular unit might be replicating differently than other units of the same hierarchical level as the result of carrying genes providing higher fitness. Active selection of a higher-unit level might result in passive selection of lower units integrated into the former one.

The values of these parameters vary in relation to the space and time in which the evolutionary trajectories and the interacting evolutionary objects are being investigated. In terms of antibiotic resistance, for instance, the parameters and evolutionary trajectories will be affected by the following factors: the local density of colonized and colonizable hosts; bacterial population sizes per host during colonization and infection; susceptibility to host colonization, including age, nutrition, and illness-facilitated colonization; frequency of inter-host interactions (such as animal-human interactions); the host's 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, and antibiotic combinations; mode of transmission of resistant organisms; transmission rates between hosts (antibiotic-treated and untreated, infected and uninfected); duration of contact between hosts; exposure to biocides; hygiene, infection control, and sanitation; food, drinking-water and water body contamination, and related host exposure; and environmental contamination by resistant organisms in soil, including sewage and water bodies.

The mere enunciation of the diversity of variable parameters simultaneously affecting the trajectories of antibiotic resistance suggests the impossibility of predicting trends. The multifactorial-based prediction of the evolution of these types of complex systems involves considerable randomness. As in weather prediction, probabilistic projections of the future can likely only be achieved for the relatively short term and for particular (well-defined) locations; however, there is room for improvement (116, 1165). In certain locations, it should be possible to find order out of chaos.

This problem has been treated in physics with the concept of the "strange attractor"(1166), which describes a set of points in a coordinate system consisting of the various parameters that affect the system, around which the system's state, plotted over time, "swirls like a ball of yarn". In other words, a limit to the unpredictability of evolutionary trajectories should be possible to trace. Chaos does not necessarily undermine the predictability of evolution under defined environmental forces (1167). The major epistemological (and technological) problem is how to combine these parameters, tracing highly complex fitness landscapes to capture all (or most) of the system's information. Reproducible causal chains and relations can be identified by multispatial convergent cross-mapping (1168), and pattern-recognition (pattern-oriented modeling) might also help in this endeavor (1169). We need a type of hyperspace landscape

geography (1170) but embedded in a time series (1171, 1172). A brilliant adaptation of these ideas to biological processes was developed by Sugihara and May (1173), who developed tools to make short-term predictions for certain nonlinear natural systems. The method is based on the accurate detection and identification of the parameter values that are represented as points in a system's attractor graph, those that are closer to the spot representing the system's present state.

Modeling Evolutionary Processes in Antibiotic Resistance

Mathematical models. A wealth of mathematical models have been developed to study the evolution of antibiotic resistance. The classic studies mostly conducted in the early 1990s (1174) were "compartmental models." The human host population is typically compartmentalized into susceptible and colonized hosts (with susceptible and resistant bacteria). The frequencies of susceptible or colonized hosts are depending on their densities, being modified by therapy (use of antibiotics, dosages and therapeutical schedules, pharmacodynamics and pharmacokinetics), prevention of transmission, and natural clearance of bacteria, including the immune response. These frequencies are measured by applying deterministic models based on a system of ordinary differential equations describing the dynamics of the densities of each type of host and the susceptible and resistant populations. Stochastic models are in most cases agent-based, in which the hosts and bacteria are tracked individually, based on the individual probability of a host being colonized by a susceptible or resistant bacterium. These models frequently employ the Monte Carlo protocol, calculating the daily probability of moving from one compartment to the other (467, 1175). Such compartmental models can be "inter-host" models, applied to the transmission of resistance between hosts, such as in the spread of resistance in hospitals or on farms, and "intra-host" models, designed to predict the emergence of resistance within the treated host (1176). These mathematical models are

mostly directed to predict the effect of targeted interventions. Similar models have been applied to study more basic problems of antibiotic resistance, such as the horizontal transfer of resistance genes in bacteria (1177). To a certain extent, mathematical approaches have helped obtain estimated "evolutionary rates", considering base substitutions through comparative studies of nucleotide sequences and the derived phylogenetic analysis (1179). Other mathematical modeling studies are based only on the "possible" structural landscapes of molecules, taking RNAs or protein molecules as variable "evolutionary units" and "fitness of phenotypes" as the replicative or enzymatic activities or their stability (628, 1180). These modeling studies do not encompass all of the complex steps and interactions of evolutionary processes, however, and require severe reductionism to be able to describe (with deterministic and stochastic modeling combinations) certain traits of the processes under study (1181).

Synthetic biology modelling evolutionary trajectories. "Long-term behavior is unpredictable" (1182). Natural complex systems increase in complexity over time, and natural complex bacterial systems (from communities of microorganisms to communities of genes), such as those involved in the evolution of antibiotic resistance, have much higher robustness to perturbations than engineered communities. Synthetic biology offers appropriate tools for the desirable reduction in the complexity factors influencing evolutionary trajectories. By developing genetic parts and devices based on transcriptional, translational, and post-translational modules, numerous genetic circuits and metabolic or antibiotic resistance pathways can be programmed in single cells, including those with a reduced genome (chassis) (28, 1183, 1184).. Synthetic biology offers a rich potential for engineering microbial consortia (1182, 1185) and, in general, natural and synthetic microbial ecosystems (1186, 1187). Until recently, synthetic regulatory networks have been designed manually; however, this limit has been surpassed

with the development of genetic circuit design automation, in which dozens of circuits can be tested in living cells, including numerous types of adaptive responses (transcriptional factors, RNA-based regulation, protein-protein interactions, and effects of recombinases) (1188).

Network analysis of evolutionary trajectories. Classic phylogenetic trees have been extensively employed to represent evolutionary processes and might clarify the historical succession (pathway) of mutational events giving rise (in the case under treatment) to a particular DNA or protein sequence involved in antibiotic resistance. In relatively simple cases, the critical steps in evolution have been predicted from the shape of genealogical trees (e.g., the influenza virus) (1189). Automated phylogenetic tools have been applied to reconstruct ancestral sequences and explore mutational trajectories (187). Inspired by bioprocess engineering, modeling framework based on flux-balance-analysis has been proposed as a mathematical method for simulating the construction of metabolism networks (1190), a method that could eventually be applied to antibiotic resistance.

However, a single genealogical tree can no longer represent the complexity of evolutionary trajectories. Trees are embedded into networks. The complexity produced by lateral gene diverging transfer among members of different lineages and introgressive merging events (120) in which elements of various evolutionary units at different biological hierarchies, and particularly among kin evolutionary units (119) interact and coevolve as composite objects requires considering “linked-trees-woods” or multidimensional super-trees, which should be constructed with networks.

Sequence similarity networks offer appropriate images of genetic diversity. However, these images do not explain the differences in similarity and the causes for divergence. Due to the advancement in network theory, a multiplicity of network analysis tools are now available, which can automatically identify composite objects formed by genetic

fragments with distinct evolutionary histories (868), representing them (with a quantitative dimension) and formally producing comparisons among an extensive number of sequences-objects. In terms of exploring the evolution of antibiotic resistance, we can deduce the possible positive or negative interactions between elements (from genes to communities and on both a horizontal and vertical axis), affecting potential evolutionary trajectories. Network analysis offers the opportunity for understanding why some evolutionary entities rarely merge or exchange traits (they can be closely related upon transmission events but without structuring a consistent association) or why others easily share common public goods. Network analysis refers to the antibiotic-resistance gene-coexistence interactions of the genes with the host bacterial genes, between mobile genetic elements interactions and relations with the host cell, and the building up of microbial communities.

For these purposes, bipartite graphs are adequate because they allow heterogeneous biological entities (e.g., plasmids and bacterial hosts) to be connected by edges (relations) (197). This type of analysis has revealed that a multitude of gene families are shared (externalized) by means of MGEs in different bacterial groups, including ultra-small bacteria (869, 1191). The analysis has enabled the identification of the "circles of species" that can be contaminated with ARGs and of the commonality of the components of these circles that will be detected by techniques such as contact metagenomics (525).

This progress in the network analysis of evolutionary trajectories has provided a new image of evolutionary trajectories (1192–1198), a rapidly growing field that now encompasses multilevel, trans-hierarchical networks that consider the evolutionary and mechanistic relations shaping phenotype-genotype maps (1199, 1200).

Computational modeling of multilevel antibiotic resistance. Antibiotic resistance evolutionary trajectories span various hierarchical levels, encompassing different

evolutionary individuals (units of selection) (61). These individuals are in some cases relatively simple (e.g., DNA fragments) and, in other cases, very complex (e.g., microbial communities). In the complex cases, there are composite evolutionary objects and composite individuals; in short, cumulative-constituted entities that require the application of ontologies and spatial-structural granularity theories (1201, 1202). Computational models are needed that consider evolution as resulting from the "independence" of each individual of the complex, heterogeneous system, with a changing set of interactions, and forming collective "independent entities", to assess (or possibly predict) the integrated evolutionary effects of all "agents" in the system as a whole (agent-based methods) (1203, 1204). In other words, there is a need for integrating intra-host and inter-host modeling to address the evolutionary epidemiology of antibiotic resistance (1176, 1177).

Membrane computing (1205) has recently made advances in the multi-level analysis of antibiotic resistance (1206). Membrane computing differs from conventional mathematical models and most computational models in representing the various actors of nested biological scenarios as particular entities (objects, "individualized" by membranes, from genes to mobile genetic elements, species, bacterial communities and hospitals). Thus, a membrane can be located inside another membrane of a higher hierarchy. Membranes are endowed with "rules" ensuring interactions with other membranes across hierarchies and mimicking evolving biological entities, given that they can independently replicate, propagate, become extinct, transfer into other membranes, exchange information according to flexible rules, mutate, and be selected by external agents (1207). Membrane computing enable s us to dissect the influence of changes in any evolutionary unit at a particular hierarchical level on the outcome of the entire system (for instance, how the plasmid conjugation rate or cellular cost compensation of harboring

plasmids influences antibiotic resistance in a hospital) (1029). Indeed, accurate modeling requires a better quantitative understanding of evolutionary processes (1208).

ECO-EVOLUTIONARY INTERVENTIONS IN ANTIBIOTIC RESISTANCE

The study of evolutionary pathways and trajectories should provide the basic knowledge to apply interventions directed to control antibiotic resistance (65, 1209). The more promising interventions directed to limiting the evolution and spread of resistance have recently been reviewed (1210) and include the following: i) reducing antibiotic selective pressures by reducing antibiotics and mobile ARGs in environments; ii) guiding antibiotic discovery to specific target selection with a low propensity for resistance evolution; iii) reducing the variation and diversification processes of resistance and reducing the mutation supply and HGT rate; iv) narrowing the window of selection of resistant variants during therapy through pharmacokinetic-pharmacodynamic optimization; v) exploiting collateral sensitivity, so that the acquisition of resistance to a drug is linked to the recovery of susceptibility to another one; vi) improving local and global healthcare practices and health policies to reduce transmission of resistant organisms; vii) increasing multiple-target therapy, including antibiotic combinations; viii) promoting antibacterial vaccination to exclude propagation of high-risk resistant clones; ix) discovering drugs acting specifically on resistant clones and selecting for drug-susceptible bacteria; and x) modulating microbiota to reduce the niches of resistant clones. In our polluted world, the pathways and trajectories of antibiotic resistance are ubiquitous; therefore, the battlefield against antimicrobial resistance is the entire microbiosphere. The global environment and therefore only global actions (global health) on significant nodal points might be able to change the rising tide of antibiotic resistance (875, 876, 1117, 1211). Interventions can be aimed in two directions: i) modifying the ecology landscapes that favor the emergence and dissemination of antibiotic resistance, essentially by controlling anthropogenic

activities and ii) developing “therapeutic approaches” to curb or slow the evolutionary processes fostering antibiotic resistance. Such an approach suggests the possibility of using “eco-evo drugs” that are not directed towards curing infections but rather by targeting resistance processes (1212).

Targeting Emergence

Until recently, the targeting emergence approach was mainly limited to evaluating the risk of resistance to new antimicrobial drugs under development. Pharmaceutical companies and even international agencies involved in the acceptation of new drugs (antibiotics) are typically satisfied with investigating the frequency of mutational resistance, not always with employing optimal methods and criteria (246). Prediction should be based on a more complete set of tests (27, 28). Novel technologies, such as advanced high-throughput genotyping, transcriptional analysis, and metagenomics, and the parallel rise of powerful bioinformatic methods are promising tools for understanding, predicting, and manipulating the evolution of antibiotic resistance (1213). Whole-genome sequencing in hypermutable bacterial organisms (including high-risk clones) challenged with antimicrobial agents might identify the more frequent (likely) genetic changes leading to bacterial resistance (322). This technology has been proposed for predicting resistance development with novel and experimental antimicrobial agents. Misjudgments as to the possibility of the *in vivo* emergence of antibiotic resistance have withdrawn or delayed the approval of useful antibiotics, as in the case of fosfomycin (1214). However, this emergence is apparently not due to the higher fitness costs of resistant mutants, at least in *P. aeruginosa* (1215). Predictions based on the detection of higher fitness resistant mutants obtained under *in vitro* serial passages in increasing antibiotic concentrations have been applied to study the mutational evolution of antibiotics (181, 193, 473).

Interventions designed to reduce mutation rates are still in the experimental phase. Spontaneous mutagenesis is a viable drug target. Research in this field is promising for controlling not only the emergence of antibiotic resistance but also tumorigenesis and resistance to anti-cancer therapy (1216). For example, RecA inhibitors block the mutational evolution of antibiotic-R (1217, 1218). The possible targets for these potential "anti-evolution drugs" are LexA (which induces the SOS in response to DNA damage), other SOS key factors, and the translocase protein Mfd (261).

The prediction of evolutionary trajectories by experimental evolution has been considered a key strategy for identifying druggable targets that could inhibit the evolution of antimicrobial resistance (1219). There is ongoing research on "evolution-proof" antibiotics, where the bacteria cannot tolerate any mechanism of resistance because there are no possible detoxifying mechanisms in nature or because these mechanisms cannot be obtained by HGT (1220). The candidates that meet these requirements include the antimicrobial peptides, including those produced by multicellular organisms as part of the innate immunity, such as peptidoglycan recognition proteins (455). Bacterial susceptibility to innate immunity proteins has been retained over millions of years.

Targeting Transmission

The control of transmission (mobility) events and processes is a key objective in the goal of limiting the spread and evolution of antibiotic resistance. Transmission acts on two levels, which we have reviewed elsewhere (194, 1221). The first is trans-acting transmission, intercellular and transhierarchical transmission, and the spatial dispersion of evolutionary units (mobile genetic elements, cells, and communities). The second is cis-acting transmission, the intracellular transmission of genetic units (sequences, genes, insertion sequences, and transposons), resulting in the creation of genetic diversity. Mobility transmission is needed to solve the problems of ecologically unfit populations,

either by the changing of patch, seeking a better (alternative) patch, or by changing the cell's adaptive resources (for instance by HGT) while basically maintaining the same type of individual. For instance, an originally susceptible clone can remain in a host population either by being readily transmitted to a nontreated host or by HGT acquisition of resistance in a treated host.

The more transmissible (or possibly endemic) clones should secure the maintenance of their susceptible populations by entering and exploiting antibiotic-free sanctuaries. Thus, interventions on trans-acting transmission should be exquisitely targeted, given that there are "healthy epidemics" of antibiotic-susceptible bacteria, some of which could be considered "under risk of extinction". One of the most promising future interventions against general host-to-host transmission is high-risk resistant clone-directed vaccination. In current practice, the prevention of transmission is mostly nonspecific ("global sanitation" in communities and "standard general precautions" in hospitals). Although undoubtedly useful, these approaches might favor the spread of the more abundant and transmissible organisms, with undesirable results if these are resistant bacteria. Control measures designed to prevent pathogen transmission and infection, such as oversanitation, might paradoxically intercept the "transmission of susceptible commensals" and increase antibiotic resistance (1206, 1222).

Interventions against cis-acting transmission are essentially still at the experimental level, although the persistence of transmissible resistance plasmids in bacterial communities is a potential drug target. Under significant antibiotic exposure, even plasmids that had a significant biological cost for their new bacterial hosts might improve their fitness, ensuring long-term persistence. This host-plasmid adaptation is partly explained by mutations in chromosomal helicases; inhibitors of the plasmid-helicase interactions might slow this adaptation (1223).

Plasmid-curing strategies were among the first to be considered, including the use of toxic DNA-intercalating agents (1224) such as acridine orange and ethidium bromide, which alter the DNA transfer between cells. A number of antimicrobials have been suggested as having plasmid-curing activity, such as novobiocin, rifampicin, 4-quinolone derivatives (1225, 1226), agents with weak antibacterial activity such as ascorbic acid (1227), and thiazine heterocyclic compounds, such as phenothiazines, which act on cell membranes and are clinically employed in psychiatric and allergic diseases (1228). Anti-HIV drugs such as abacavir and azidothymidine have also been shown to reduce interbacterial plasmid transfer (1229, 1230).

Specific approaches have been developed to reduce plasmid conjugation, developing conjugation inhibitors to fight the spread of ARGs among bacteria (936, 1231). Tanzawaic acids are polyketides with anti-conjugation properties (1232). Unsaturated fatty acids and alkynoic fatty acid derivatives, such as 2-hexadecanoic acids (most importantly, 2-bromopalmitic acid) likely act to reduce the frequency of conjugation by influencing the association with bacterial membranes of TrwD, a member of the secretion ATPase, which is required in the conjugation process (1233).

Other approaches are being investigated with the aim of limiting plasmid curing and transmission, including the use of CRISPR/Cas-based approaches (1234, 1235) and plasmid interference, based on plasmid incompatibilities and toxin-antitoxin “addiction” systems (1236).

Despite these strategies, the most effective method for reducing transmission is to reduce contact between global resistance units, such as hospitals, farms, and even countries. Antibiotic-resistant bacteria are regularly released in water through the human/animal stools. Unless that water is treated, which is uncommon in various areas of the world, these bacteria can freely spread. Basic sanitation procedures, such as wastewater

5268 treatment, and social norms to prevent unnecessary contact are still the main elements in
5269 reducing the transmission of antibiotic resistance (136, 875).

5270 **Targeting Restoration of Susceptibility**

5271 A number of the strategies discussed in the previous section might contribute to the
5272 elimination of antibiotic resistance. A number of other strategies focus directly on ARGs,
5273 such as CRISPR-based anti-resistance antimicrobials (1237), The restoration of
5274 antibiotic-susceptible bacterial populations constitutes a main objective to contain
5275 antibiotic resistance. The possibility of reversion depends on the ability of susceptible
5276 populations to outcompete the resistant ones ("selection of the susceptible"); however,
5277 "where do susceptible genotypes that replace resistant lineages come from?" (1238).
5278 Could we alter in our favor the elements and conditions of such competition or at least
5279 coexistence? We should first maintain the diversity of susceptible populations as a basic
5280 requirement; their extinction would make restoration impossible. This is, however, a
5281 highly unlikely outcome; there is a balancing selection in heterogenous bacterial
5282 populations, recurrent reverse evolution, with heterogeneous fitnesses in fluctuating
5283 habitats, different niches and subniches, and different population sizes, promoting the
5284 coexistence of sensitive and resistant strains (1239). We can then modify co-selective and
5285 antagonistic pleiotropy effects, fitness costs of resistance, and compensatory adaptations
5286 (585, 1238). The third step is to exploit the epistatic mechanisms (e.g., those influencing
5287 membrane biosynthesis and transport) and chaperones creating a method for disrupting
5288 the evolution of antibiotic resistance (1240) or restoring susceptibility, altering intrinsic
5289 resistance mechanisms (1241, 1242). The fourth step is to imagine "ecological
5290 interventions" to alter the conditions in which competition takes place. Resource
5291 limitation prevents the emergence of drug resistance by intensifying intra-host

competition (1243). Presumably, this limitation also ensures bacterial coexistence (susceptible and resistant populations) in the gut microbiota (1244, 1245).

Reestablishing microbiota-mediated colonization resistance after antibiotic therapy could markedly reduce infections, particularly those caused by antibiotic-resistant bacteria. Ongoing studies are identifying commensal bacterial species that can be developed into next-generation probiotics to reestablish or enhance colonization resistance (1246). Several studies based on fecal microbiota transplantation offer promising results to eradicate multidrug-resistant organisms from the gut (1247). Phage therapy can be useful for eliminating particular drug-resistant clones from the microbiota (1248, 1249).

ANTIBIOTIC RESISTANCE AS A MODEL PROBLEM OF THE INFLUENCE OF ANTHROPOGENIC EFFECTS ON THE BIOSPHERE

Microbial pathways and trajectories involving antibiotic resistance occur in a changing biological world influenced by anthropogenic activities, resulting in the reduction of diversity of certain species but likely also fostering speciation (1250). Humans create and select environments, and there is a reciprocal selection between biological entities and environments.

Anthropogenic Antimicrobial Agents in the Biosphere: Meta-selection of Antibiotic Resistance

Antibiotic resistance is not only a threat for the treatment of infections in humans and animals. The massive environmental pollution with antibiotics, biocides, heavy metals, and numerous other anthropogenic substances able to select bacterial populations that host ARGs can alter the biosphere's natural ("healthy"), microbial community

composition. Local ecological conditions modulate antibiotic resistance (e.g., resistance in *Pseudomonas* is higher in the water of tropical areas than that of temperate areas) (1251) and will influence global ecosystem processes, including effects on microbial primary producers, carbon dioxide respiration and decomposition, nitrogen cycling, photosynthesis, chemosynthesis, heterotrophic production, and biodegradation. As a primary example, phytoplankton consists mostly of cyanobacteria, responsible for more than 25% of the total free oxygen production and carbon dioxide fixation. Cyanobacteria are susceptible to widely employed antibiotics; however, it has been shown that cyanobacteria might contain class-1 integrons containing *sul1* genes, which might serve as capturing units for resistance genes (876, 1252, 1253). The second example is the effects of antibiotics on the soil and particularly in the bacterial rhizosphere, essential not only to the maintenance of nitrogen fixation and plant health but also to the phyllosphere (the microbial colonizers of stems, leaves, flowers and fruits), which is endowed with its own resistome (1254). Interestingly, endophytic bacteria might evolve local adaptive mechanisms, resulting in antibiotic resistance (1255). Antibiotics affect plants, even at low concentrations, leading to delayed germination, lower biomass allocation, and less diversity (1256). A similar situation occur with insects, particularly those dependent on bacterial endosymbionts (1257, 1258), altering the healthy gut microbiota, as is the case with honeybees (1259). Protistan composition in soil is affected by antibiotics (1260), as is the biology of nematodes (1261). Even mitochondria and chloroplasts could acquire antibiotic resistance (1262, 1263). As a final example, antibiotics probably play a role as signaling agents in nature (a type of ecological hormone), linking various microbial communities, and likely interacting with higher entities (363). The effects of anthropogenic antibiotics should be understood, considering the extensive use of other

environmentally-released biocides, such as herbicides and insecticides, which frequently kill the microorganisms' hosts and the microorganisms themselves (876).

From the above examples, antibiotics might alter the biosphere's local equilibrium, which can be recovered by acquiring antibiotic resistance. Plants, nematodes, and insects hosting resistant bacteria will benefit in terms of reproduction over those that maintain susceptible organisms and will therefore select for microbial evolutionary pathways and trajectories resulting in resistance. Rhizobacteria, plants, nematodes, protozoa, and insects are eco-biologically linked. Any deleterious antibiotic effect in one of them will therefore influence the health and possibly the evolution of the entire system. In short, the exposure of biosphere ecosystems to antibiotics will select for antibiotic resistance, in a type of higher-order selection or meta-selection.

Antibiotics, Antibiotic Resistance, and the Evolution of the Microbiosphere

Evolution can be defined as the flow of life, ensuring replication of chemical and biological entities over time. Time is constantly offering discontinuities that should be overcome by microbes. In the our part of the time arrow, anthropogenic activities have created a multitude of discontinuities, pushing bacteria out of equilibrium with their environment. The human production and dissemination of antibiotics, which leads to ecological damage and antibiotic resistance, is a quintessential "One Health and Global Health" issue (876, 1264). Antibiotic (anti-life) compounds interfere with the existence of microbial organisms, and antibiotic resistance is a force (process) ensuring the continued flow of life under antimicrobial exposure, a force based on gaining information to resist, a force translatable to a gain in energy (1265), pushing the altered bacterial world to reacquire order, equilibrium, and life and to oppose the entropic effect of antimicrobial agents. This force fuels the evolution of genes, genomes, and coordinated ensembles of genes and genomes. Beyond the changes due to errors and accidents, the "read-only-

memory" model (1266) progresses by the continuous expression of "read-write" restructuring of informative storage mechanisms (1267), allowing changes without disturbing the long-term (evolutionary) integrity of the microbial world.

Directional evolution promoted by antibiotic natural selection should reduce entropy and randomness (1268). However, the never-ending diversification of genes and lineages suggests the possibility of an "*ex unum pluribus*" entropic evolution (1269). We should once again remember that biological systems appear to be subjected to an ineluctable tendency to progress evolution, resulting in complexification. By natural selection, antibiotics might reduce the diversity but not necessarily the complexity of the microbial world. Evolution not only creates entities of higher hierarchical levels (such as eukaryotic cells, plants animals), thereby making it more complex, it also produces complexity inside individual entities. The diversification inside each family of ARGs (generation of orthologs) and inside each bacterial clone of a single species is also a process of complexification, which increases the number of genes (143, 1270) and, in the jargon of genome complexity metrics, sequences of length K (*k*-mers) (1271). The important issue is whether such an increase in endo-diversity under antibiotic exposure, which facilitates the emergence of novel genetic associations and epistatic bonds, is fostering more evolvability or evolutionary energy (1272).

EVOLUTION OF ANTIBIOTIC RESISTANCE: A GLANCE FROM PHYSICAL SCIENCES

Evolutionary processes are increasingly considered in theoretical physics. An approximation between a "more biologized physics" and a "more physicalized biology" could provide important epistemological benefits. At the start of this review, we distinguished pathways as sequences of changes (such as mutational changes) forming chains in which each step facilitates the next and favoring, step by step, a significant

increase in a particular process leading to antibiotic resistance. The sequence is not fixed (there are alternatives for including successive links in the chain), but the number of possibilities is relatively limited (the gene-protein space of variation). These "logical" and, to a certain extent, "reproducible" chains of events depend on the biological context in which they evolve; in that regard, evolution is always contingent. Trajectories are boosted by these pathways, but pathways do not determine the direction of the trajectories, which have a considerably higher degree of freedom, given that they depend on complex contingent ensembles of biological entities (mobile genetic elements, clones, species, and communities), whose own evolution spaces (located on endless gradients of niches and variable environments) are frequently subjected to stochastic events.

In other words, pathways constitute the more rigid parts of the evolutionary trajectories. In a previous review (180), we compared the intrinsic indetermination of evolutionary trajectories with the dynamics of a multiple pendulum/oscillator (1273). Imagine a string with almost contiguous beads (the pathways) but embedded in bodies (biological entities) that are linked to others by free-swiveling strings or ball-joints (multi-body pendulum), (Figure 9). Each of these bodies can take different directions (the trajectories), eventually stochastically linking with other bodies and exchanging or complementing their pathways, which was described above as "cord" or "spinning" trajectories. The chaotic disaggregation of trajectories generated by the multiple pendulum is somewhat compensated by this type of networking. Most importantly, the pathways pushing evolutionary trajectories are evolving with the trajectory itself, which has been described as the simultaneity of evolution and the evolutionary solutions (1274). Lastly, pathways and trajectories of antibiotic resistance constitute a complex chemical-physical reaction-diffusion system in which substances (biological entities) react and are transformed into each other, which results in diffusion, causing their spread (1275).

FINAL CODA: ABOUT THE INTELLIGIBILITY OF EVOLUTIONARY TRAJECTORIES OF ANTIBIOTIC RESISTANCE

Knowing the limits of our endeavor to discover laws of the natural world is an obligation of science. Our knowledge and the possibility of communicating our findings to future generations depends on the rational structure of our proposals. Rationality requires the existence of a certain order in the interaction among the elements involved in the process under study or at least some solid probabilistic associations; chaos cannot be explained(1272). In this review, we examined the plethora of processes involved in antibiotic resistance. Evolutionary pathways are composed of logical sequences of events in the acquisition of resistance and, despite their diversity, can frequently be faithfully reproduced under controlled evolutionary experiments. However, evolutionary trajectories depend on an unlimited number of stochastic events influencing myriads of interactions among a hierarchy of nested biological elements, from proteins to genes to species and communities, each acting in a selfish manner, running on their own evolutionary trajectories and colliding and collaborating with other evolutionary trajectories. In the case of complex evolutionary trajectories, certain trends can be assumed by accurate and long-term observations; however, such trends only apply for shorts periods (as with weather prediction, which also deals with highly complex systems). As in the rest of the biological sciences, our understanding and our intelligibility of the evolution of antibiotic resistance has a part that is logical, demonstrable and based on solid information and a part that is based on undetermined information, which we can attempt to predict based on observations. However, this perception of reality (the experience of seeing) can have a quality, almost as a logical thought (1276). Therefore, if the knowledge of evolution is composed by thinkable and only showable parts, and a

strategy of half-thinking, half-seeing is needed to make intelligible the evolutionary processes, including antibiotic resistance (1277). We are obliged to continue our daily tasks to ascertain the details of the multi-hierarchical interactions among entities involved in antibiotic resistance, in the hope that, in the future, complex computational models and artificial intelligence tools can help push the frontiers of our knowledge, to understand and control the negative influence of antibiotic resistance on medicine: One Health, and Global Health.

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AUTHOR BIOGRAPHIES

Dr. Fernando Baquero, MD, PhD, Graduate in Medicine at the Complutensis University in Madrid, specialized in Clinical Microbiology and doctorate in 1973 at the Autonomous University in Madrid. Postdoctoral Courses at the Pasteur Institute, Paris (1973-1974). From 1977 to 2008, Director of the Department of Microbiology at the Ramón & Cajal University Hospital in Madrid. Scientific Director of the Ramón & Cajal Health Research Institute (IRYCIS) (2008-2015). From 2008, Research Professor in Microbial Evolution and Director of the Division of Microbial Biology and Evolution of Microorganisms at IRYCIS. He has been working on the biochemistry, genetics, and

evolution of antibiotic resistance during 40 years, maintaining from 1995 a close interaction with the Department of Biology, Emory University. More than 500 publications in peer-reviewed journals, including several books (Evolutionary Biology of Bacterial Pathogens, ASM Press), he obtained the ICAAC-ASM Award (2000), and is member of the European and American Academy of Microbiology.

Dr. Jose L. Martinez, Chemist by formation, Microbiologist by career. He was Research Fellow at the Department of Microbiology at the Ramón y Cajal Hospital, Madrid, and the Imperial Cancer Research Foundation (UK). Currently Full Research Professor at the National Biotechnology Center of the Spanish Council for Scientific Research (CSIC), leading the laboratory of Ecology and Evolution of Antibiotic Resistance. His research focuses on the molecular bases of antibiotic resistance and the effect of acquiring resistance in the virulence and the overall physiology of bacterial pathogens. He is particularly interested in the use of predictive approaches for studying the emergence of resistance as well as on the role that natural (not clinical) ecosystems may have in the origin, evolution, and transmission of antibiotic resistance.

Dr. Jerónimo Rodríguez-Beltrán studied Biology at the Autonomous University of Madrid and earned his Ph.D in Molecular Microbiology at the Institute of Biomedicine of Seville (Spain) in 2015. During his PhD, he focused on understanding how recombination and mutation contribute to the development of antibiotic resistance. In 2016, he joined the division of Microbial Biology and Evolution at the Ramón y Cajal Institute for Health Research (IRYCIS) in Madrid as a Postdoctoral fellow to study the evolution of plasmid-mediated antibiotic resistance. As a result of his work, he received the Ippen-Ihler Memorial prize to the best young investigator on plasmid biology. After a research stay at the Pasteur Institute (Paris), he has established his research group at IRYCIS. His research interests focus on understanding the molecular mechanisms that

fuel bacterial evolution with the aim of developing new strategies to counter the evolution of antibiotic resistance.

Dr. Juan-Carlos Galán PharmD; PhD, studied in Complutensis University, Madrid; specialist in Medical Microbiology from 1997, he reached the doctoral degree in 2002 in this University (genetics of beta-lactamases in anaerobes). Staff member in the Microbiology Department of Ramón y Cajal hospital in 2011, in charge of the area of Virology and Molecular Biology, and Coordinator of the Ramón y Cajal team included in the Center for Network Research in Epidemiology and Public Health (CIBERESP) of the National Institute of Health of Spain. He has been actively working in bacterial hypermutation, phylogeny of bacterial and viral species, and experimental evolution, mainly to reconstruct the evolutionary trajectories of genes involved in antimicrobial resistance. At present time, his interest is focused on the framework of multiple gene variations involved in the evolution of gene interactions, including antibiotic collateral susceptibility.

Dr. Alvaro San Millán (D.V.M., Ph.D.) is a Group Leader in the National Centre for Biotechnology in Madrid. Doctorate on plasmid-mediated antibiotic resistance at the Complutensis University of Madrid in 2010. During his Ph.D, he complemented his training with several stays at the Pasteur Institute in Paris. As a postdoc, he worked for four years at the Department of Zoology of the University of Oxford, studying the evolutionary bases of plasmid-mediated antibiotic resistance. In 2016, he started his research group at the Department of Microbiology at Ramon & Cajal University Hospital in Madrid, where he analyzed the evolution of plasmid-mediated antibiotic resistance in the patients and the hospital setting. In 2020, Alvaro joined the Spanish National Center for Biotechnology as a Tenured Scientist in Plasmid Biology. Alvaro is interested in

understanding the role of plasmids as catalysts of bacterial evolution, with a special focus on the evolution of plasmid-mediated antibiotic resistance in clinical settings.

Dr. Rafael Cantón, PhD, studied Pharmacy at Complutensis University, Madrid (Spain) and obtained his PhD degree in 1994. He was trainee as Clinical Microbiology Specialist at the Microbiology Department at the Ramón y Cajal University Hospital in Madrid (Spain) in which he is currently the Head of the Department since 2011. He is also Associated Professor at the Complutensis University. His research activity on antimicrobial resistance, novel techniques on antimicrobial susceptibility testing and chronic respiratory tract infections (mainly in bronchiectasis and cystic fibrosis) is developed within the Spanish Network for Research in Infectious Diseases (REIPI, http://reipi.org/) and Institute Ramón y Cajal for Health Research (IRYCIS, http://www.irycis.org) in which he coordinates the Microbiology, Immunology and Infection Area. He has been Chairman of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and President of the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC). He has published more than 500 articles in peer-review journals.

Dr. Teresa M. Coque, Ph.D., FISAC, graduated as a Pharmacist and Clinical Biochemist, and received her PhD in Medical Microbiology from the Complutensis University of Madrid (Spain). A long postdoctoral training (1993-1997) at the University of Texas at Houston (USA) gave her background on molecular epidemiology and genetics of antibiotic resistance. She is a Senior Scientist at the Ramón & Cajal Institute for Health Research in Madrid. Her focus is based on studying the ecology and the evolution of opportunistic bacterial pathogens and mobile genetic elements involved in the transmission of antimicrobial resistance for the last 25y. Advanced -omics applied to the analysis of bacterial populations dynamics is her research interest nowadays. She

published about 170 papers, special issues and chapters on the field, and serves on the editorial boards of several journals. She is/has been member of international committees (JPIAMR, WHO, EFSA) and evaluation grant panels related to antimicrobial resistance.

TABLE and TEXT OF FIGURES

Table 1. The components shaping pathways and trajectories in the evolution of antibiotic resistance.

Evolutionary **objects** are the biological substrates, from proteins to microbiotas, on which evolutionary **processes** act, producing phenotypes, whose frequency is governed by **evolutionary mechanisms**, which are under the influence of **evolutionary** drivers (41, 61, 1278, 1279).

Evolutionary objects	**Evolutionary processes**
Antibiotic molecular targets	Growth
Antibiotic transporters	Mutation
Single and supra-protein domains	Genetic diversification
rRNA sequences	Epigenetic epistasis
Intrinsic resistance genes	Fitness cost and cost compensation
	Gene amplification

Regulators of antibiotic transporters and resistance genes

Stress-response networks

Acquired resistance AbR genes

Non-coding segments of genome

Random chromosomal sequences

Genes with epistatic relations with AbR

Contingency loci

Operons

Insertion sequences

Small intergenic repetitive sequences

Gene cassettes

Integrons

Transposons

Plasmids

Integrative-conjugative elements

Genetic islands

Bacteriophages

Bacterial species

Gene conversion

Gene redundancy

Gene promiscuity by HGT

Gene recombination

Genes insertions and deletions

Genes silencing

Gene degeneracy

Gene decontextualization by HGT

Promoter recombination

Genome recombination

Gene(s) conjugation

Gene(s) transformation

Gene(s) transduction

Transfer by extracellular vesicles, nanotubes

MGE* transmission

MGE-host interactions

MGE mobilization

MGE recombination

Bacterial subspecies	MGE copy number
Bacterial clones (genomotypes, STs)	MGE maintenance
Clonal ensembles	MGE incompatibility
Genetic exchange communities	Bacteria-bacteria contacts and recognition
Metagenomotypes (i.e. enterotypes)	Bacterial antagonism, cooperation
Resistomes (intrinsic and mobile)	Inter-host transmission
	Host-bacterial interactions
	Microbiota coalescence

*MGE: mobile genetic elements

Evolutionary mechanisms	**Evolutionary drivers**
Selection by other reasons than AbR	Bacterial stress
Selection dependent on AbR	Bacterial bottlenecks
Cross-selection	Human antibiotic consumption
Co-selection	Animal antibiotic consumption
Selection in antibiotic gradients	Agricultural antibiotic consumption
Random drift	Historical antibiotic use
Random draft, Gene hitchhiking	Antibiotic pharmacokinetics/dynamics

Neo-functionalization-Exaptation	Collateral susceptibility and resistance
Founder effects	Human and animal age, health and nutrition
Persistence	Bacterial transmission
Tolerance	Hygiene, Sanitation,
Inducibility of AbR	Crowded human or animal populations
Resilience in the presence of Ab	Water and sludge reuse
Changes in fitness	Antibiotics and biocides in the environment
Niche exploitation and co-exploitation	Pollution with heavy metals
Niche construction	Environmental pollution with human and animal bacteria
Habitat compartmentalization	Decrease in animal and global biodiversity
Spatial structuration	Environmental variation
Transmission, dispersal	Global warming
Clonal shifts, clonal waves	Social norms for the use of antibiotics
Clonal bunch selection	Social norms for environmental health
Reticulation of evolutionary trajectories	

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FIGURES:

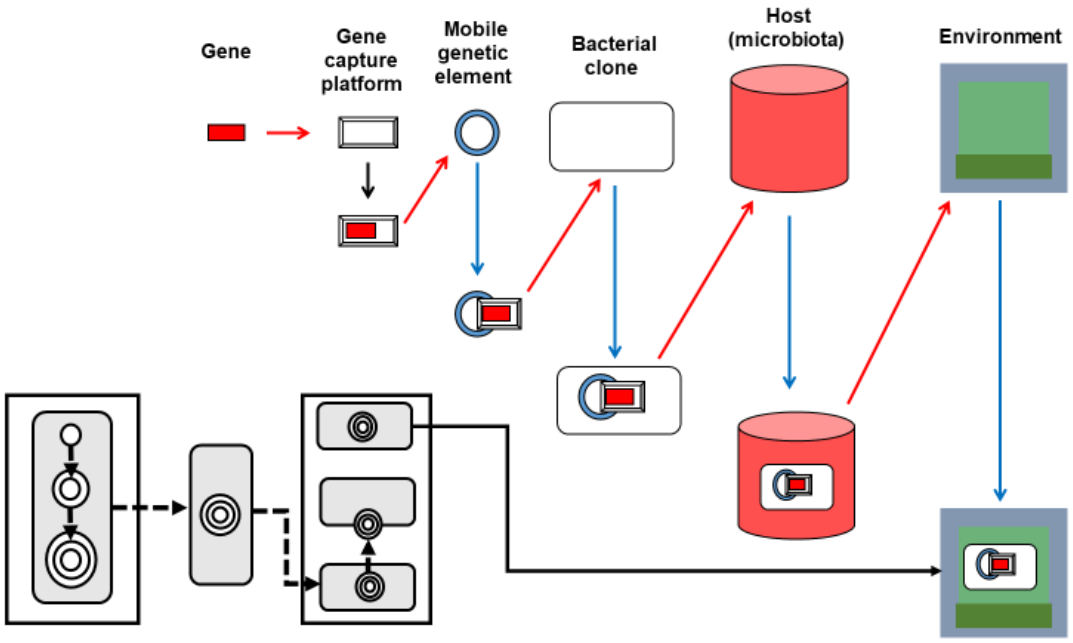

Figure 1. **The basic nested structure of the evolutionary units involved in antibiotic resistance**. From left to right, a resistance gene is caught by a gene capture platform (as an integron), which might in turn be inserted into a conjugative mobile genetic element (as a plasmid), which is acquired by a particular bacterial clone. This clone is inserted in the host microbiome; the host is part of an environment where the resistance gene contributes to the environmental resistome. As shown in figure 2, evolutionary units are units of selection, i.e., they can be independently selected. The small figure in the bottom right shows that all of these successive steps are due to internal (cellular) cis-acting transmission events (resulting in concentric rings), followed by unenclosed trans-acting transmission events (clone with resistance plasmid, host-microbiota, environment); for example, when a bacterial cell containing a plasmid and a gene (concentric rings) is transmitted from a human host to another host and then to the environment (black line) (194, 1221).

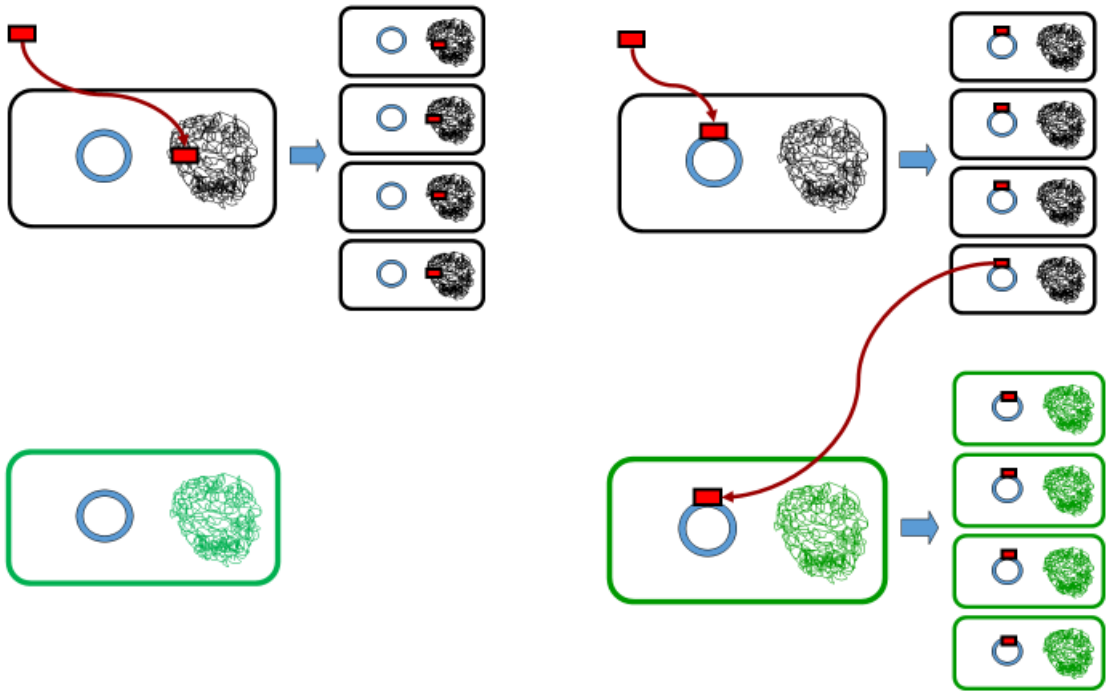

Figure 2. **Units of selection as evolutionary units.** A bacterial cell and a conjugative plasmid carrying antibiotic resistance genes constitute different evolutionary units, given that they are independent beneficiaries. At the top, a resistance gene that is externally acquired (small red rectangle) by the cell can be integrated either in the chromosome (black string ball) or in a conjugative plasmid (blue ring). In a selective event, the cell with the red gene in the chromosome reaches 4 copies, but the plasmid is independently transferred to a different bacterial cell (green), which is also selected and reaches 4 copies. At the end, the balance for each type of cell is 4 copies, with 8 copies for the plasmid, indicating that, under this single selective antibiotic event, the plasmid is a better beneficiary than any of the other bacterial cells hosting it; in other words, the plasmid is an independent unit of selection, a different evolutionary unit.

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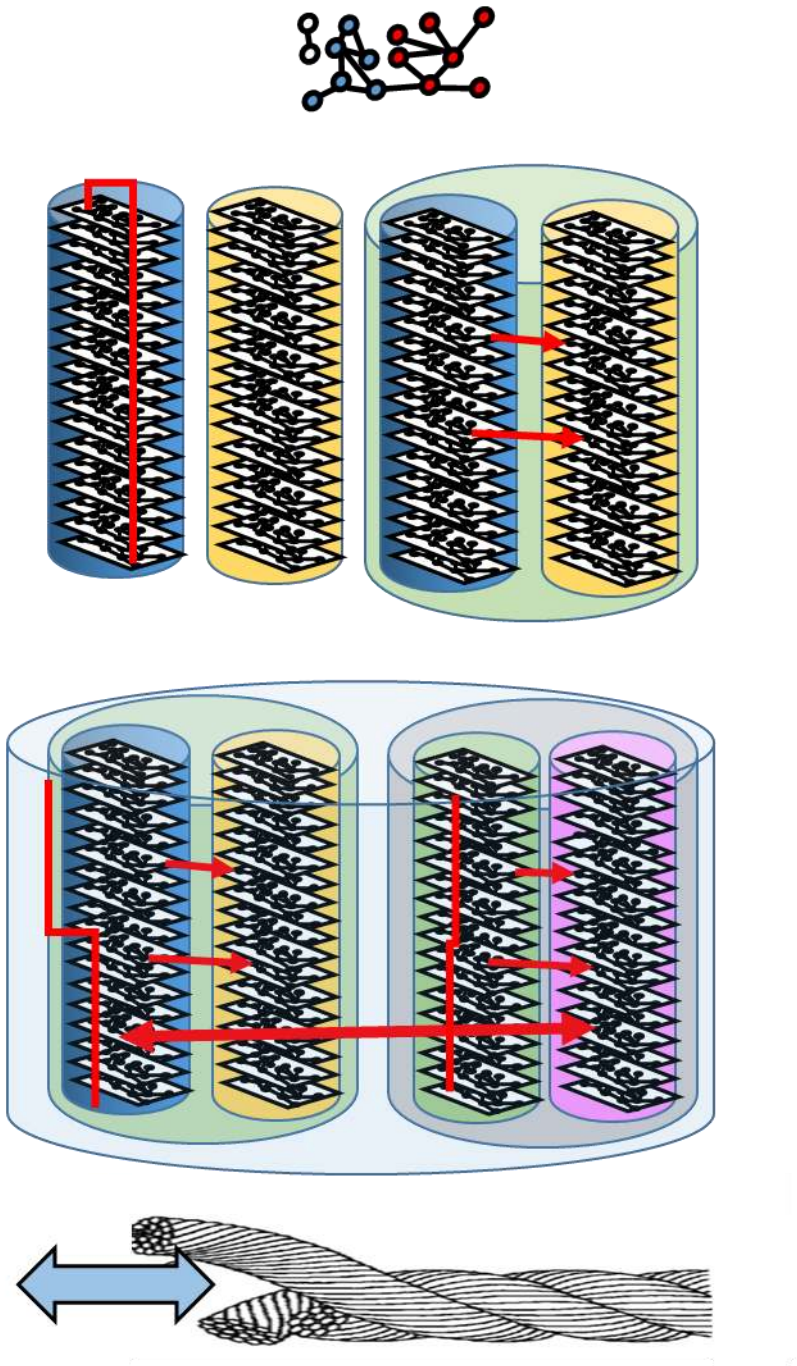


Figure 3. The topological interactions of bacterial populations in space and time: from clones to spinning evolutionary trajectories. Bacterial species have a complex population structure consisting of clonal ensembles linked by phylogenetic relations, which can be represented as a network in a plane (top of the figure). These clonal ensembles are sequentially maintained (top to down in the cylinders), but there is the possibility of clonal variation or recombination over time (red vertical arrow). The structure of each bacterial species is

frequently in the neighborhood of other species with their own structure. This vicinity is represented by a larger cylinder consisting of both of the species (mid-section of the figure) and enables horizontal genetic interactions (horizontal red arrows). In complex ecosystems (such as microbiota), several cylinders are ecologically and functionally integrated, facilitating genetic exchange among apparently distant lineages (lower section). The interactive spinning of different evolutionary strands results in a single evolutionary material, which can be represented as a rope, based on vertical and horizontal interactions (red lines), giving rise to twisted common trajectories; however, the components can eventually be untwisted in changing environments (bottom of the figure). The concept depicted here is that the events resulting in antibiotic resistance not only influence the trajectory of a particular clone or species in which they emerge but also the trajectories of complex bacterial ensembles.

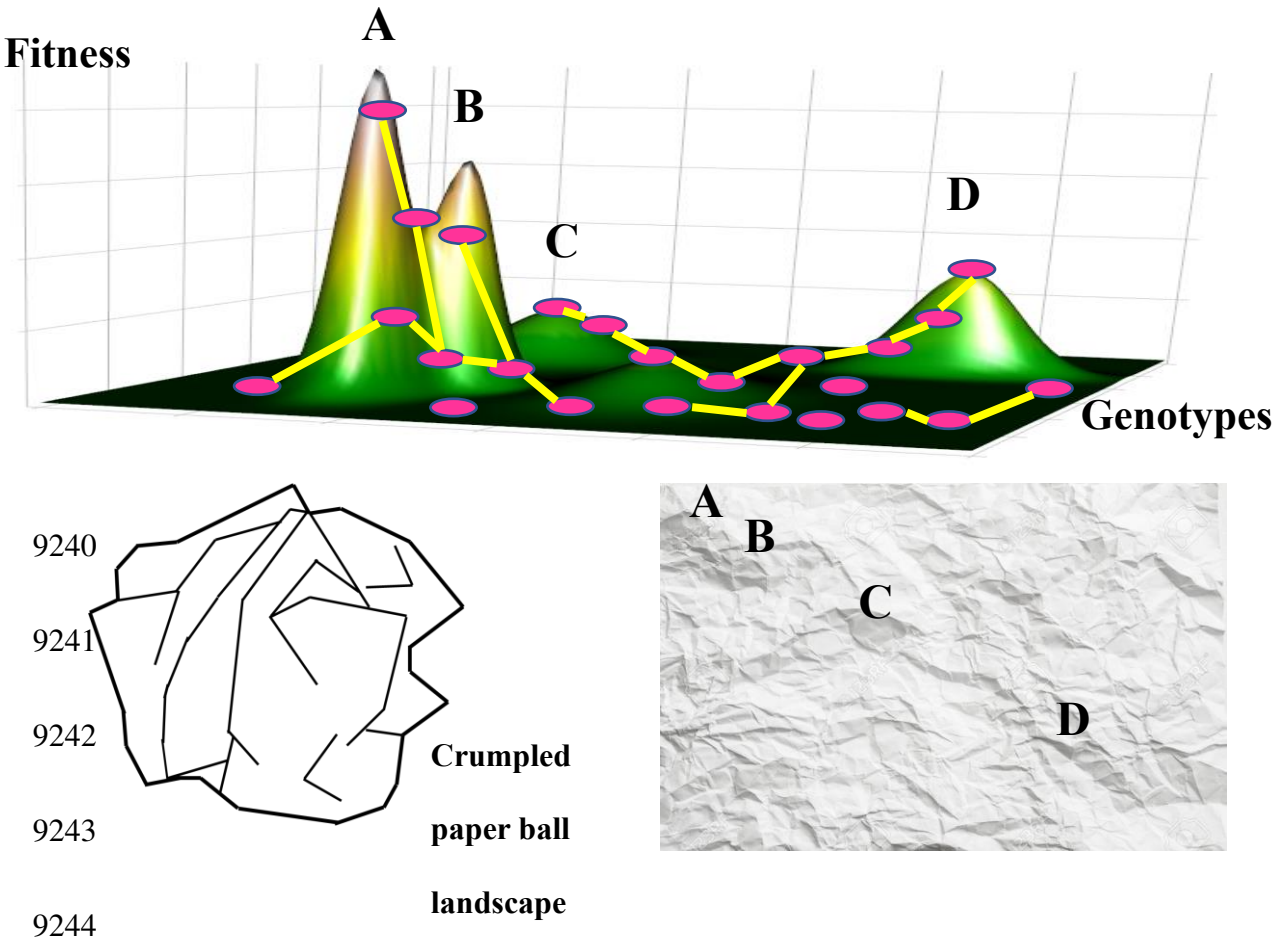
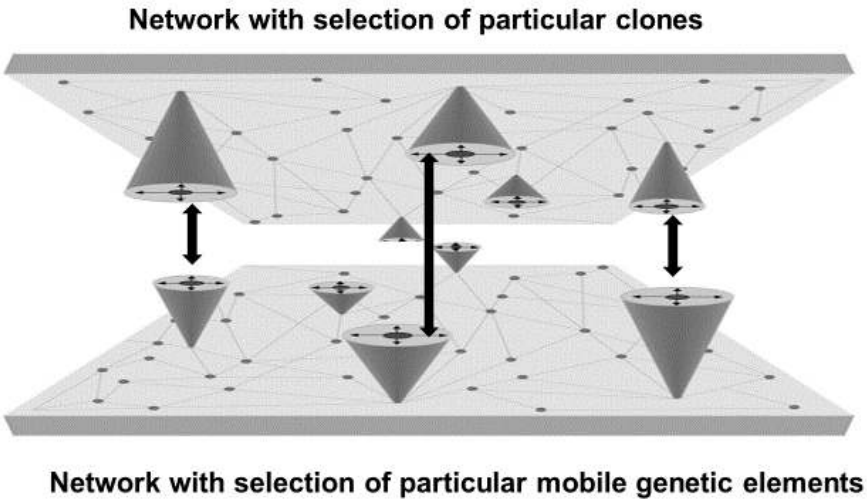


Figure 4. Fitness landscapes in antibiotic resistance. On the top, an image of the classic fitness landscape metaphor was developed in 1932 by Sewall Wright, where in a bidimensional plane (black in the figure) different genotypes are represented, their corresponding "height" in the vertical axis showing the fitness of each genotype (reproductive success) under the conditions of the landscape. Red ovals correspond to the variation (for instance mutation) from one genotype to another one (yellow lines). Note that series of mutations (pathways) might reach low (C), medium (D) or high (A,B) fitness peaks (for instance reaching very high MICs), but some of these pathways might have been originated just by random drift (without natural selection) in the flat área of the landscape. If this landscape is crumpled as a paper ball (down, left), peaks can go into proximity, and the genotype selected into a peak can have access to other fitness peaks (eventually resulting in genetic recombination or exchange). Down-right, the deployement of the paper ball to illustrate the fitness landscape.



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9259 **Figure 5**. **Interactions between evolutionary networks.** The top and bottom horizontal
9260 fields depict networks where the respective bacterial clones and mobile genetic elements
9261 (MGEs) harboring resistance genes evolve independently. In each of the network planes,
9262 there are selection events, amplifying the clones or MGEs (cones). Occasionally, a
9263 successful plasmid interacts with a successful clone (two headed arrows), eventually
9264 creating a high-risk resistant clone. This figure is inspired by the classic figure by Feil
9265 and Spratt (1280).

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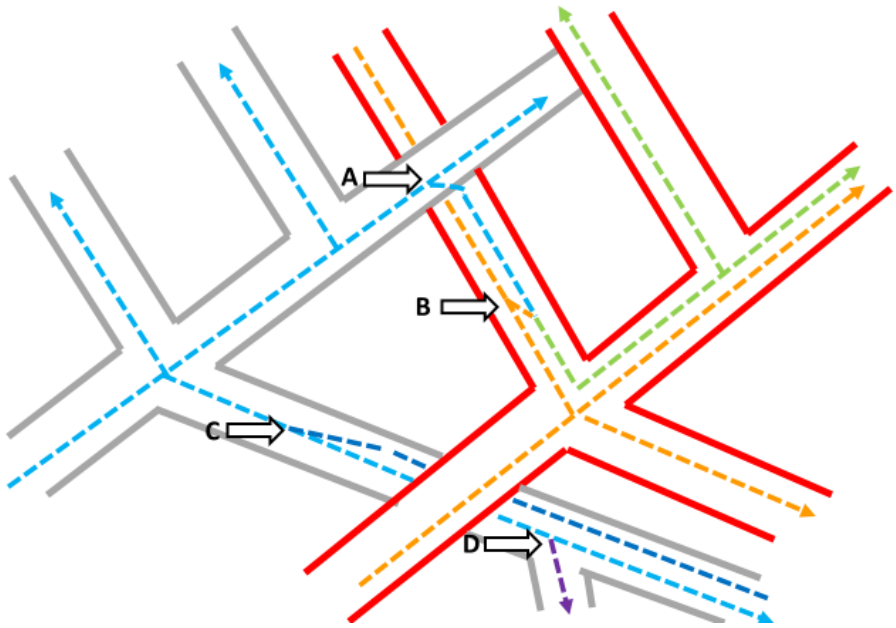

Figure 6. **Phylogenetic networks and antibiotic resistance: trees within trees.** Two separated phylogenetic networks (grey and red, either from clones, species, families) are superimposed. Inside the branches, the mobile genetic elements (MGEs) carrying antibiotic resistance genes co-evolve with their hosts. Arrows represent the various events that modify the evolution of MGEs: A) The light blue MGE introgresses (i.e., conjugates) from the grey to the red tree; B) The recombination with the indigenous MGE (yellow) creates a new MGE variant (green), which eventually evolves within a separate branch of the red tree; C) A variant (dark blue) of the indigenous (light blue) MGE of the grey tree emerges (mutation, internal recombination). This variant can segregate into a new branch of the grey tree. The figure's purpose is to show the mixture of MGE-bacterial associations and the eventual modifications of their co-evolution, giving rise to novel MGEs able to colonize other bacterial branches.

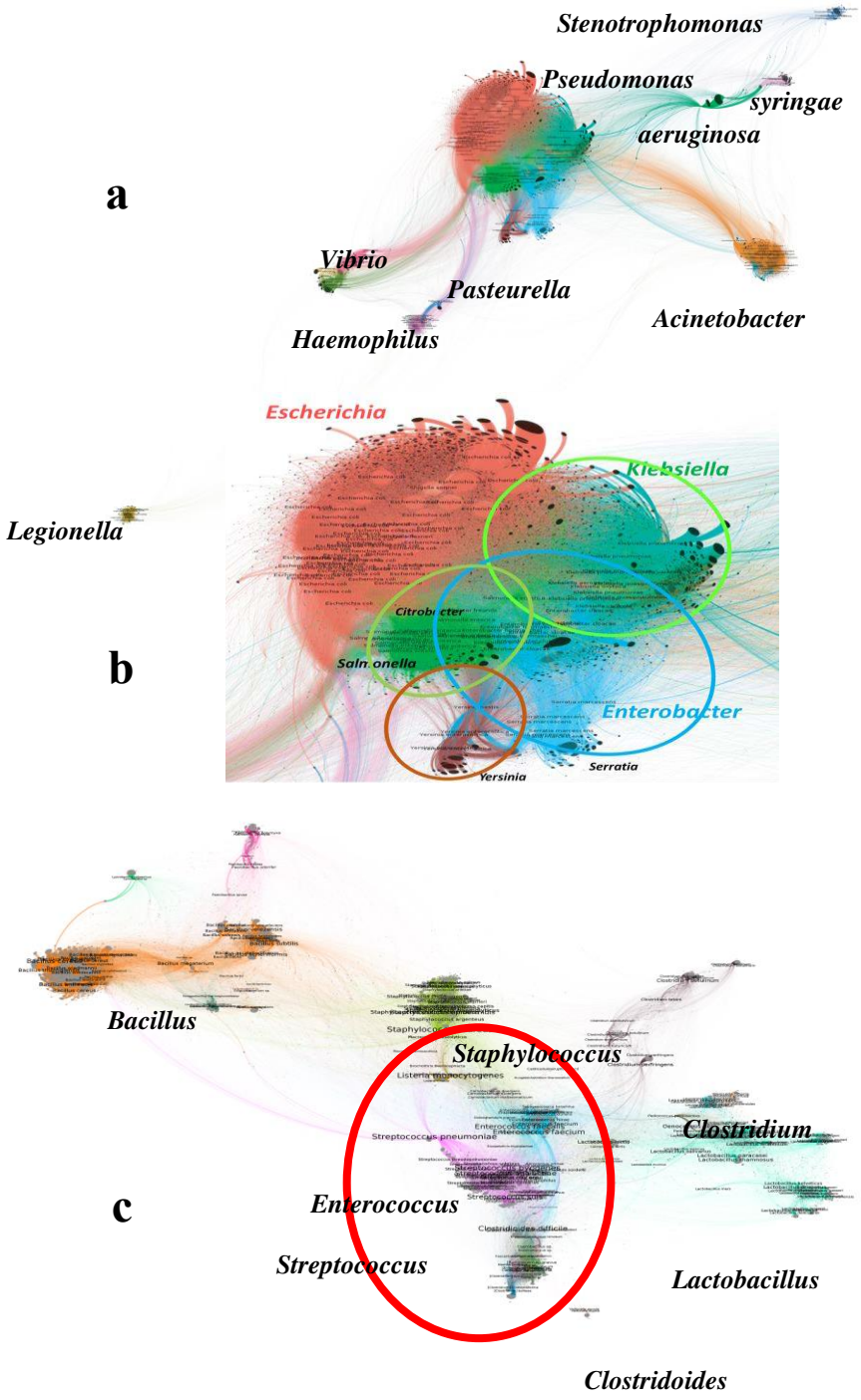
a
Stenotrophomonas
Pseudomonas
syringae
aeruginosa
Vibrio
Pasteurella
Haemophilus
Acinetobacter
b
Escherichia
Klebsiella
Legionella
Citrobacter
Salmonella
Enterobacter
Yersinia
Serratia
c
Bacillus
Staphylococcus
Listeria monocytogenes
Streptococcus pneumoniae
Clostridium
Enterococcus
Streptococcus
Lactobacillus
Clostridioides

Figure 7. **Flow of accessory genes among bacterial species should correspond to the flow of accessory genes**. This figure presents bipartite networks illustrating the accessory gene (protein) flow among species (genus) of the major taxons of Gamma-Proteobacteria (a,b) and among Firmicutes (c). Connections between two bacterial species indicate that the same accessory gene is shared, and the distance between the species (genus, in italics) is proportional to the number of connections. (b) Detail of the "core" of Enterobacteriaceae species sharing accessory genes; "trumpet-like" patterns on the surface of some clusters correspond to accessory genes that are unique for a particular strain (not connected with any other). The colored circles in (b) indicate the blurred borders of the species more frequently sharing accessory (and resistance) genes in Gamma-Proteobacteria and the "core" group exchanging accessory (and resistance) genes in Firmicutes.

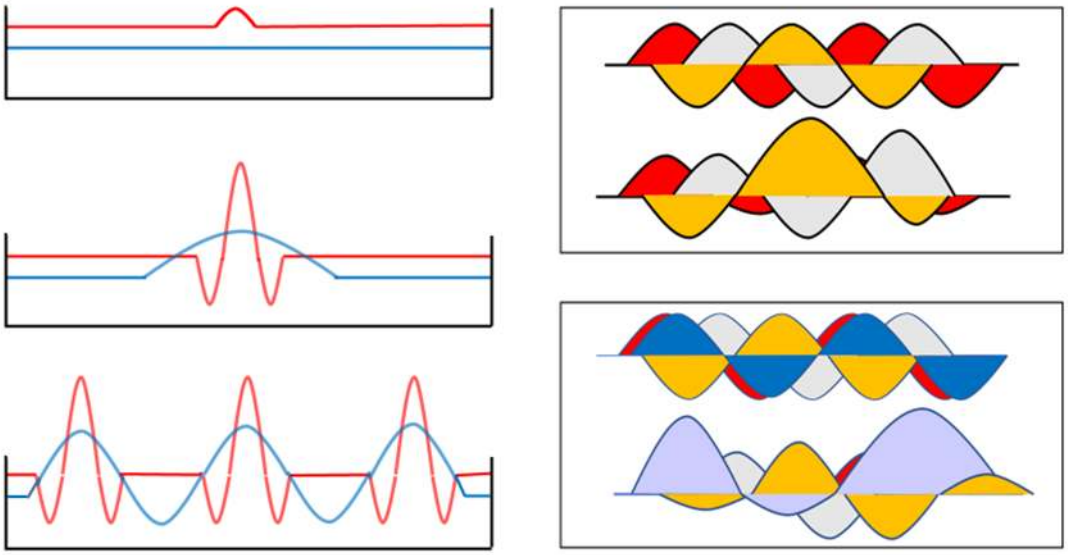

Figure 8. Populational (clonal) fluctuations and antibiotic resistance. On the left, the equilibrium between the red and blue subpopulations is locally perturbed, occasionally due to the local antibiotic selection of a recently acquired resistance trait (or a local adaptive advantage), giving rise to wave dynamics recalling a Türing instability (1180, 1281). The local selection of the red population influences the blue one, which might start competing with the red, creating an expansion of instability, giving rise to new fluctuations in the equilibrium of both the red and blue populations. On the right in the upper box, three populations or clones (colored red, yellow, and white) fluctuate in a given environment (as the microbiota). Eventually the yellow population is selected, altering the other populations. In the lower box, the simultaneous selection of the blue and red waves results in a merging, with the emergence of a new and predominant population, a super-clone (1282), as might occur in environments exposed to aariety of antibiotics. The main concept represented here is that antibiotics contribute to the instability of the clonal structure of bacterial populations, giving rise to dominant waves that can spread across the environment.

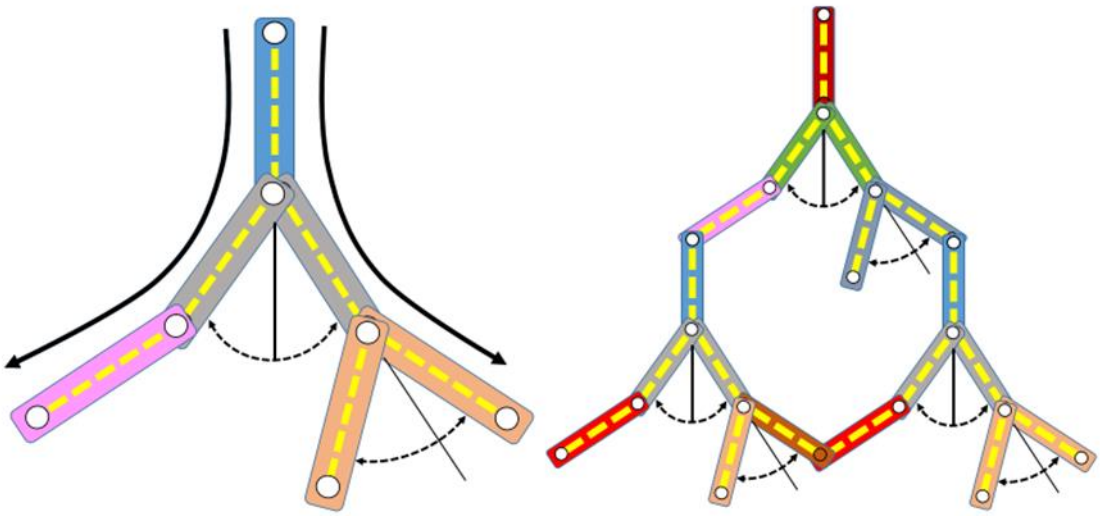

Figure 9. Evolutionary pathways and trajectories. On the left, adaptive pathways and trajectories are represented as parts of a multibody pendulum, with mobile rigid members hinging on each other. The rigid parts represent the pathways, formed by broken yellow elements, corresponding to series of successive events (as mutations) leading to an efficient resistance phenotype, which are predictable and reproducible to a certain extent in the laboratory. However, these rigid parts located in bacteria oscillate by moving in different environments, where they can approach and be linked by swivel joints (white circles, representing mobile genetic linkages) to other organisms. An immense number of possible trajectories are thereby created, each offering new possibilities for interaction and linkage with other rigid parts, again eventually mediated by mobile genetic elements (the ball joints). The resulting multiple pendula greatly increases the indetermination of trajectories, approximating a chaotic behavior, with diversifying kinetics (black arrows). On the right, the possibility of loop formation among the trajectories is presented, providing a certain rigidity (and thus potential predictability) to the system. Note that the rigid parts might correspond to various units of selection, organisms, supraorganisms (such as species) and suborganisms (such as plasmids), which create a highly complex evolutionary frame.

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