ANNUAL REVIEWS

Annual Review of Microbiology

Evolutionary Trajectories to Antibiotic Resistance

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Annu. Rev. Microbiol. 2017. 71:579–96

First published as a Review in Advance on July 11, 2017

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-090816-093813

Keywords

epistasis, mutation rates, population bottlenecks, relative fitness, coselection, selection pressure

Abstract

The ability to predict the evolutionary trajectories of antibiotic resistance would be of great value in tailoring dosing regimens of antibiotics so as to maximize the duration of their usefulness. Useful prediction of resistance evolution requires information about (*a*) the mutation supply rate, (*b*) the level of resistance conferred by the resistance mechanism, (*c*) the fitness of the antibiotic-resistant mutant bacteria as a function of drug concentration, and (*d*) the strength of selective pressures. In addition, processes including epistatic interactions and compensatory evolution, coselection of drug resistances, and population bottlenecks and clonal interference can strongly influence resistance evolution and thereby complicate attempts at prediction. Currently, the very limited quantitative data on most of these parameters severely limit attempts to accurately predict trajectories of resistance evolution.

Contents

1. INTRODUCTION

Evolution can be influenced by unexpected factors and is therefore often thought to be unpredictable. In contrast, much of the research applied to public health is based on the implicit belief that microbial evolution leading to infectious diseases and antibiotic resistance is predictable and therefore can be controlled so as to prevent clinical problems. To perform such predictions, we need to further develop theory and methodological tools and determine values for the main parameters influencing resistance evolution. Below we discuss some of the biological parameters that need to be better defined to allow the development of a predictive approach to antibiotic resistance.

How antibiotic resistance mechanisms emerge, spread, and are maintained in a population of bacteria is determined by the interplay of several basic factors, such as (*a*) mutation supply rate, (*b*) level of resistance conferred by the resistance mechanism, (*c*) fitness of the antibiotic-resistant mutant bacteria as a function of drug concentration, and (*d*) strength of selective pressures (each discussed in Section 2). In addition, epistatic interactions and compensatory evolution, coselection of drug resistances, and population bottlenecks and clonal interference can strongly influence resistance evolution and thereby complicate attempts at prediction (discussed in Section 3). Numerous epidemiological factors (not discussed here), including host population biology and immunity and infection controls, also influence the spread and maintenance of resistance in the host population.

A graphic illustration of resistance evolution is mutational space, i.e., all types of mutations or HGT events that can generate a specific drug resistance (**Figure 1**) and their characteristics with regard to rate of appearance, level of resistance conferred, and fitness in relevant environments as a function of selective pressure. It is obvious from **Figure 1** that from the point of view of risk of resistance evolution, if the mutations/HGTs are in the lower left-hand corner, then resistance evolution will be slower as compared to if they are located in the upper right-hand corner. In principle, if we had a complete knowledge of this mutational space and how selection pressures vary over time we could predict which resistant mutants would be likely to emerge and spread. However, at present we have very limited knowledge of the required parameter values, effectively prohibiting such prediction.

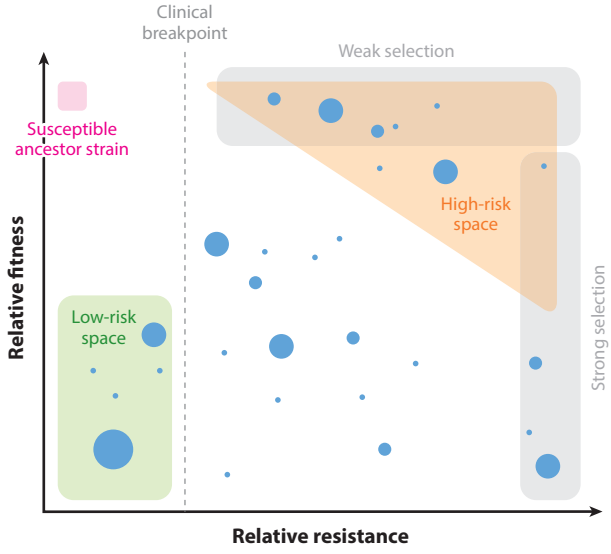

Figure 1

Mutational space. This plot shows all types of mutations or horizontal gene transfer events that can generate a specific drug resistance and their characteristics with regard to rate of appearance (lower or higher rates of appearance are illustrated as blue circles of smaller or larger size), level of resistance conferred, and fitness in relevant environments as a function of selective pressure. If we had complete knowledge of the mutational space and how selection pressures vary over time, we could predict which resistant mutants would be likely to emerge and spread.

2. MUTATIONAL SPACE

2.1. Mutation Supply Rate

Mutation supply rate is determined by population sizes and rates of mutation and HGT, and the extent of genetic heterogeneity in a bacterial population present in human hosts is largely influenced by the mutation supply rate and the population dynamics of both bacteria and hosts (123). Generally, we have poor knowledge of bacterial population sizes within infected hosts, but in the few cases where population size is known, it is generally high enough to suggest that resistant mutants are present in the population at any given time point (see below). Population sizes during major urinary tract infection can approach 10^{10} per bladder; in respiratory and wound infections in animal models bacterial counts can exceed 10^{10} per gram of tissue (108); for pulmonary tuberculosis infections in humans the number of bacteria per milliliter of sputum can be 10^5–10^6 (89) and even higher inside a lung cavity (25, 26); and during meningitis and bronchitis bacteria can be present at a concentration of 10^9 per milliliter (15, 40).

Mutation rates have been measured in bacteria (41, 71), and they are typically $\leq 10^{-10}$ per nucleotide per generation (1, 37), resulting in genomic mutation rates (e.g., in *Escherichia coli*) in the order of 0.003 mutations per genome per generation (36). In contrast, rearrangement mutations (insertions, deletions, duplications, inversions) can occur at much higher rates, with, for example, duplications forming at rates of 10^{-5} to 10^{-3} per cell per generation (95, 96) and with steady-state frequencies of duplications for any given gene in the range of 10^{-5} to 10^{-2} (3). Similarly, HGT frequencies (i.e., bacterial conjugation) can vary from essentially nondetectable

($<10^{-9}$) to as high as 50% in 10-min conjugational matings (38), depending on transfer mechanism, bacterial species, and transfer conditions (23).

Measurements of in vitro rates of mutations against current clinical antibiotics span from 10^{-11} to 10^{-5} per cell per generation when selection occurs at drug concentrations greater than two times the minimal inhibitory concentration (MIC) for the susceptible wild-type strain. In the lower range (10^{-11} to 10^{-10} per cell per generation) we find oxazolidinone resistance in *Staphylococcus aureus* (68) and in the higher range (10^{-5} per cell per generation) mecillinam resistance in *E. coli* (114). Interestingly, in spite of this million-fold variation in in vitro resistance mutation rate, the burden of resistance in clinical settings, with regard to rate of emergence and frequency, is not correlated to these mutation rates in any obvious way. This lack of correlation can be explained by the above numbers where the total bacterial population size in an infected individual typically is $\gg 10^{10}$ and mutation rates $>10^{-10}$ per cell per generation. This implies that the probability of fixation of a resistance mutation is not strongly limited by the mutation rate because preexisting resistant mutants are often present in the infecting bacterial population when treatment is initiated. Thus, our focus on rates of resistance mutations as a predictor of risk of resistance development is probably misguided, and instead risk predictions ought to be based on other parameters (4).

2.2. Level of Resistance

A key factor determining the success of a resistant mutant is its level of resistance, which can vary extensively depending on the resistance mechanism and the conditions under which resistance is measured. Typically, the level of resistance is measured by disk diffusion tests, broth microdilution, or E-tests to obtain the MIC. Even though MIC determinations are easy, cheap, and rapid, they also lack sensitivity; they are only static end point determinations. Certain resistance mechanisms result in miniscule increases that can only be detected in time-kill experiments (but not with traditional MIC determinations) (70), whereas other mechanisms provide resistance levels that are higher than the water solubility limit of the antibiotic (e.g., *rpsL* mutations in *Salmonella typhimurium* give streptomycin resistance >15 mg antibiotic/mL) (116). As a rule, mechanisms that involve reduced uptake/increased efflux confer a lower of level resistance than those that modify the target or inactivate the drug itself, but there are many exceptions to this generalization. Resistance mechanisms can also be distinguished with regard to how bacterial growth is affected when antibiotic levels are increased. For certain mechanisms growth is completely unaffected by increasing drug concentrations, whereas with other mechanisms growth monotonically decreases until it stops at the MIC (47, 82, 114). Thus, the relative fitness (see below) of an antibiotic-resistant bacterium might be constant or vary extensively depending on antibiotic concentration, which needs to be taken into account when making predictions (29). Another complicating factor during prediction is how measurements of resistance in the laboratory (typically performed in rich oxygenated medium with bacteria growing exponentially in a planktonic state) can be extrapolated to resistance levels of the bacteria in a host or the environment. Thus, recent work has shown that a substantial number of resistances are strongly dependent on environmental and growth conditions such that the environment can strongly modulate and alter the phenotypic effect of the resistance mutation/gene. Such modulation might occur by a variety of mechanisms, for example, collective bacterial interactions or alterations in bacterial physiology due to the presence of specific metabolites/growth conditions (52, 62). It will be a challenge to define these environmental dependencies and incorporate them into models and predictions.

2.3. Fitness

The vast majority of the experimentally examined resistance mechanisms result in reduced fitness (relative to a susceptible ancestor), as measured by growth and survival under different conditions, but exceptions exist where resistance appears neutral or even beneficial (6, 9, 51). The relative fitness of a drug-resistant bacterium, both in the absence and in the presence of the drug, is also the key parameter in determining its evolutionary success in host populations and other environments. Thus, the rate of emergence, the steady-state frequency of resistance at a given selective pressure, and the rate of reversibility when the selective pressure is reduced are largely determined by the fitness costs of the resistance mechanism, as shown by theoretical work, epidemiology, and laboratory experiments (6, 9, 122, 124). A key question is to what extent fitness measurements made in the laboratory can be used to predict the evolutionary success of different resistance mechanisms in clinical settings. Based on the studies that have been performed, fitness measurements made in the laboratory (e.g., exponential growth rates in rich media) appear to have clinical relevance in that they concur with epidemiological studies of the prevalence of resistance alleles in clinical isolates. For example, in clinical isolates of *Mycobacterium tuberculosis* and in *Staphylococcus aureus* the most commonly found rifampicin resistance mutations are those that confer the lowest costs under laboratory conditions (20, 87). Similarly, for aminoglycoside-resistant *M. tuberculosis* the more fit mutants were also the most common types found in patients (18, 103). These and a later study (105) also demonstrated clearly that the mutants most commonly encountered clinically and with the highest fitness also had a high level of resistance, illustrating that the most successful clones are those where high-level resistance can be acquired with minimal or no loss in fitness (upper right-hand corner in **Figure 1**).

2.4. Selective Pressures

With regard to prediction of resistance evolution, selective pressure might be the most difficult factor to determine and incorporate into models. Thus, in humans, animals, and other environments, bacterial pathogens are exposed to a wide range of various selectors that often are present in complex mixtures (e.g., antibiotics, biocides) and whose concentrations may vary extensively over time. As a result, the strength of selection is generally very difficult to determine outside of a laboratory setting. In addition, the selection process for resistance will vary considerably depending on whether drug concentrations are high enough to (*a*) prevent pathogen growth (lethal selection if >MIC, nonlethal selection if <MIC) and (*b*) allow growth of both susceptible and resistant bacteria (7). In the former case, resistant mutants need to exist before the application of selection, and the rate of enrichment is determined by the number of mutants in the population and their fitness at the specific antibiotic concentration. However, during a nonlethal sub-MIC selection mutants may emerge, with a rate of enrichment determined by the number of mutants in the population and the fitness difference between susceptible and resistant cells. Thus, as illustrated in **Figure 1** and shown by recent studies, the rate of emergence and the type of mutants selected differ between lethal and nonlethal selective pressures (7, 46, 47, 88). At high lethal antibiotic concentrations, rare preexisting mutations of large effect that provide high-level resistance in one genetic event tend to be selectively enriched (right-hand side of **Figure 1**). In contrast, at nonlethal antibiotic concentrations, the enriched mutants usually result from many mutations of small effect on resistance (but which combined might generate high-level resistance). Importantly, the weaker the selection (i.e., the smaller the fitness differential between susceptible and resistant bacteria) is the stronger the enrichment is for mutants with low fitness cost (upper part of **Figure 1**).

Furthermore, because of the stepwise selection of successive small-effect mutations at weak nonlethal selection pressures, mutator strains that have an increased probability of acquiring resistance by mutation or HGT are enriched (7). Paradoxically, weaker nonlethal selection might thus enrich for more problematic mutants (high-fitness strains and mutators) than lethal selection.

3. EPISTASIS, COSELECTION, AND OTHER COMPLICATIONS

Although the trajectory of antibiotic resistance evolution could, in principle, be predicted based on a complete knowledge of (*a*) mutation rates, (*b*) level of resistance, (*c*) impact of each mutation on relative fitness, and (*d*) selection pressure exerted on the organism by an antibiotic, in reality there are additional factors that significantly influence the trajectory of resistance evolution and complicate predictability. Below we discuss the influence of epistasis, coselection, and transmission bottlenecks in bacterial populations in shaping the evolutionary landscape of antibiotic resistance.

3.1. Epistasis and Compensatory Mutations Affect Trajectories of Resistance Evolution

Epistasis describes situations where a phenotype associated with a gene allele can differ depending on its genomic context (**Figure 2*a***). For example, a mutation might confer antibiotic resistance with a particular fitness cost, but the cost might be reduced by the presence of a mutation in a separate gene. The term epistasis encompasses the types of examples that have been extensively described in classical genetic studies on suppressor mutations affecting mRNA translational fidelity (45, 98). Epistasis increases the difficulty of predicting phenotypes after horizontal transfer of genes into novel genetic environments. Epistasis is very common and may have a major impact on the trajectory of antibiotic resistance evolution (14, 34, 61).

Epistatic interactions could affect the level of resistance (the MIC), the frequency of resistance, or the relative fitness of a resistant mutant (**Figure 2*b*,*c***). Epistatic effects on the level of resistance can be easily understood by comparing the effects of antibiotics assayed in biochemical assays versus their effects when measured in whole-cell assays. For example, many potential antimicrobial compounds have been identified in high-throughput screens based on their affinity for, and inhibition of, a purified cytoplasmic drug target, but whether that high affinity translates into a low MIC value usually depends on the activities of other genes, in particular those that influence cell wall structure and compound influx into and efflux out of the whole cell (48, 107). The phenotype of resistance associated with target mutations can also be significantly influenced by the interplay between the activities of multiple genes. For example, in *E. coli*, resistance to fluoroquinolones caused by target mutations in topoisomerases is almost completely dependent on the bacteria having a functioning drug efflux system (84). The explanation is that reduced affinity for the target is insufficient to confer clinical resistance when the intracellular drug concentrations are much higher, as they become in the absence of efficient efflux. Selections made in vitro also clearly show that epistasis can reduce the fitness costs of mutations conferring resistance to different classes of antibiotics, including streptomycin, rifampicin, fusidic acid, and ciprofloxacin (19, 21, 63, 74, 104) (**Figure 2*b***). Such epistatic interactions affecting the fitness of resistance mutations are common and have been observed in different species, including *E. coli* (10, 60, 74, 115), *S. typhimurium* (17, 73), and *Pseudomonas aeruginosa* (49). Concerning streptomycin and rifampicin, resistance is caused by mutations altering a component of a multiprotein complex (protein S12 in the ribosome, and the β subunit of RNA polymerase, respectively), and the epistatic compensatory mutations affect either the same or a second protein component of the same complex. In these examples the original fitness cost is incurred because the resistance mutations reduce the kinetic efficiency of

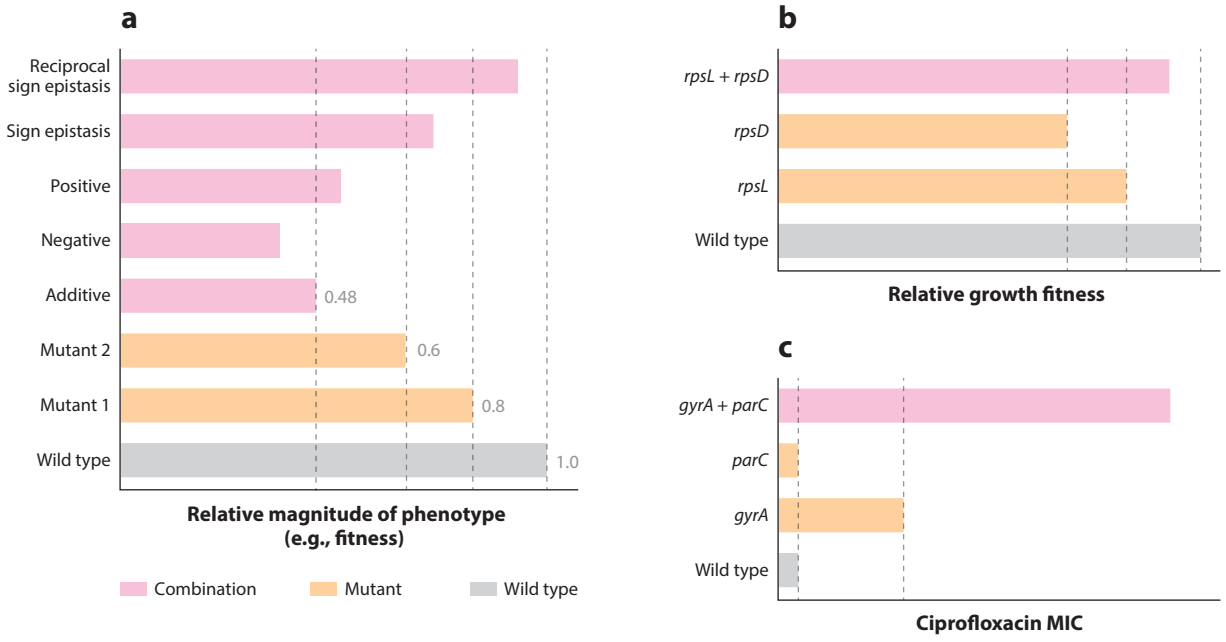

Figure 2

Epistasis constrains evolutionary trajectories. (*a*) General outline of terminology used to describe effects of epistatic interactions between different genes/mutations. The interactions are epistatic if the magnitude of the phenotype of the double mutant is not a simple product of the magnitude of the individual mutant phenotypes. Take fitness as an example to define the terminology: Negative epistasis means that the double mutant has a lower fitness than predicted by additivity, positive epistasis that the double mutant has a higher fitness than that predicted by additivity but lower than that of each single mutant, sign epistasis that the double mutant has a higher fitness than one of the single mutants, and reciprocal sign epistasis that the double mutant has a higher fitness than each of the single mutants. (*b*) Resistance to streptomycin caused by mutation in ribosomal protein S12 (*rpsL*) often comes at the cost of reduced growth fitness. Epistatic mutations in other ribosomal protein genes (e.g. *rpsD*) restore fitness to near-wild-type levels, but by themselves are also low-fitness mutations. (*c*) Resistance to ciprofloxacin in clinical isolates of *Escherichia coli* is typically associated with mutations in both *gyrA* and *parC*. However, while a single mutation in *gyrA* causes a significant increase in minimal inhibitory concentration (MIC), a single mutation in *parC* has no effect at all on MIC. The double mutation (*gyrA* + *parC*) shows reciprocal sign epistasis causing a large increase in MIC, much greater than predicted from the magnitudes of the individual phenotypes.

the ribosome or the RNA polymerase, machines that are critical for supporting maximum growth rate. The compensatory mutations in each case restore kinetic efficiency without causing loss of resistance. Where it has been tested, the compensatory mutations by themselves (in the absence of the resistance mutation) also reduce fitness. Consequently, these double mutants are examples of epistasis that exemplify, depending on the specific mutations involved, positive epistasis, sign epistasis, or reciprocal sign epistasis (**Figure 2*b*,*c***), where the relative fitness of the double mutant is greater than the expected product of the fitness of the individual mutants.

A potential consequence of epistatic genetic interactions is that they can potentially direct evolution along predictable trajectories. For example, in *S. typhimurium*, streptomycin can select the mutation *rpsL* K42T/N, and the phenotype conferred will be very-high-level resistance but with relatively low growth (16). The future evolutionary direction of such a mutant, in competition with the wild-type in the absence of drug selection, is likely to be toward extinction. However, if it is rescued by the acquisition of a fitness-compensatory mutation, that mutation is most likely to occur in one of several genes coding for other ribosomal proteins, including *rpsD* and *rpsE* (16). This knowledge provides a high degree of predictability, at least as far as the next step in the

evolution of the streptomycin-resistant mutant. Accordingly, understanding the details of epistatic interactions can be used to predict likely evolutionary trajectories, as illustrated by the extensive experimental evidence on the influence of fitness costs of resistance on the trajectory of resistance evolution (49, 74, 99, 119, 120). Although the term epistasis usually refers to phenotypes involving different genes, it can also be applied to the interactive effects of mutations within a gene (56, 80). Accordingly, studies of evolution of β-lactam resistance in vitro show that mutations within a single *pbp* gene often show strong epistatic interactions. This constrains the order in which mutations arise and creates a situation where the first mutation to arise strongly affects the subsequent evolutionary trajectory (100, 121).

Epistasis also plays an important role in maintaining antibiotic-resistance plasmids in bacteria. Frequently when plasmids first enter a naive host they reduce fitness, and subsequent genetic changes affecting both the host chromosome and the resistance plasmid can reduce these costs, enhancing plasmid persistence (32). Mutations enhancing plasmid persistence have been associated with positive epistasis (69) and reciprocal sign epistasis (106). Positive epistasis between different plasmids in one host has also been shown to reduce the costs associated with carrying multiple plasmids, and with increasing the stability of small, individually costly, nonconjugating plasmids (101, 102). The recombination of DNA sequences (e.g., transposons, ICE elements, or prophage) into a bacterial chromosome or the creation of a larger-scale hybrid bacterial chromosome [as happened with *Klebsiella pneumoniae* ST258 (28)] will be subject to rules of selection and epistatic interactions similar to those that apply to the acquisition of plasmids. The expectation is that acquired foreign DNA, although it may be positively selected in some environments because it carries, for example, genes causing favorable virulence or resistance properties, may also interfere with cell physiology, causing reduced relative fitness of the recombinant, at least in some environments. Transcriptional silencing of foreign DNA, mediated by proteins like H-NS, may reduce the magnitude of associated fitness costs (12, 83), potentially allowing more time for strains that acquire foreign DNA to adjust through the acquisition of mutations in both the host genome and the newly acquired DNA.

An interesting variant of epistasis affecting antibiotic resistance is so-called collateral sensitivity. This term describes a situation where the development of resistance to one antibiotic is associated with increased susceptibility to another antibiotic (2, 42, 72, 90, 93, 110, 113). Large-scale studies of collateral sensitivity (55, 59, 64, 92) show that it is a common phenomenon, although in most cases the mechanism is not understood. One case in which the mechanism has been explained concerns vancomycin-resistant MRSA (methicillin-resistant *S. aureus*). These bacteria are susceptible to a combination of vancomycin and oxacillin but are resistant to each drug individually (93). The sensitivity in this example occurs because the oxacillin-resistant PBP2a enzyme does not process the D-Ala-D-Lac precursors of peptidoglycan synthesis associated with the vancomycin-induced *vanA* operon. The result is that in combination these antibiotics inhibit cell wall synthesis.

In summary, epistasis acts to constrain and direct evolutionary trajectories, and a deeper understanding of epistatic interactions would improve predictability of the evolutionary trajectories of strains that are newly antibiotic resistant.

3.2. Coselection Phenomena

Coselection, in contrast to epistasis, refers to situations where one resistance gene hitchhikes with another gene, usually because they are physically linked to one another. Typically this means that the two genes are colocalized on the same chromosome or plasmid, but it would also apply if, for example, one gene were located on a plasmid that in turn was closely associated with a chromosome carrying the other gene (**Figure 3**). Smaller genetic units where coselection of

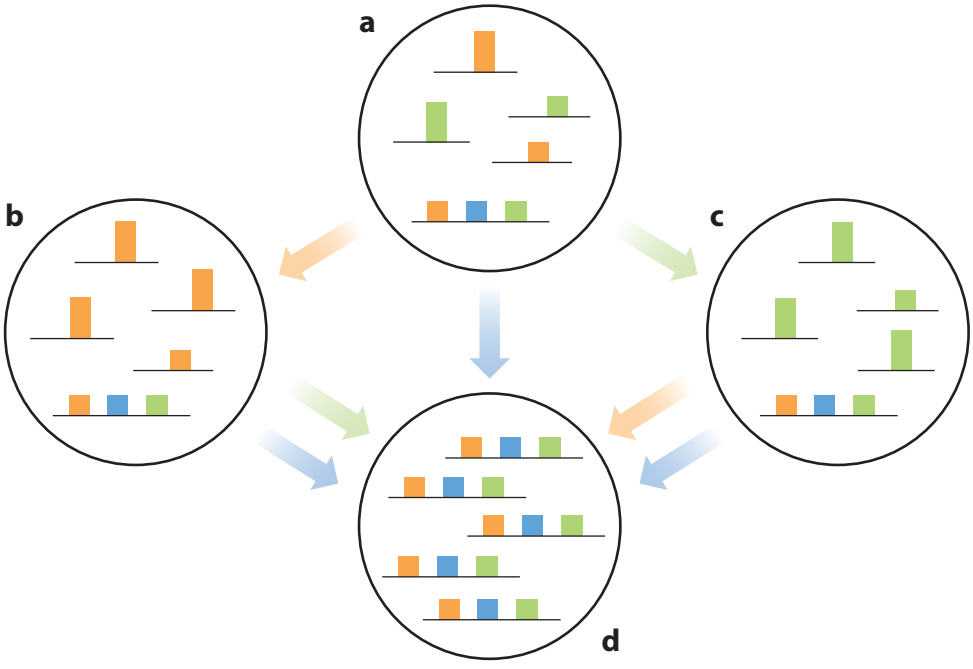

Figure 3

Coselection affects evolutionary trajectories. Colored bars represent genes with different selectable phenotypes (*orange*, *green*, and *blue*). The height of a bar represents its intrinsic fitness (resistance level, growth fitness, virulence, transmission, etc.). Arrows represent different selection pressures (e.g., antibiotics). (*a*) Population of bacteria with different phenotypes. (*b*,*c*) Selection for either orange or green selects for the co-linked genes, in addition to the gene directly selected. (*d*) Selection for blue, or successive different selections enrich for organisms that carry co-linked, selectable genes. Each selected gene in panel *d* is not necessarily the best variant from the original population in panel *a*, but these variants are good enough and succeed because of their genetic linkage to other selected phenotypes.

resistance genes can occur include mobile genetic units like transposons (97), and genetic units that collect genes, like integrons and superintegrons (24). The importance of coselection as a phenomenon affecting resistance to antibiotics is clear from even a cursory examination of plasmids carrying resistance genes. Almost all resistance plasmids carry multiple antibiotic resistance genes, and in many pathogen species multidrug resistance is synonymous with plasmid carriage (22, 78, 79). The consequence of genetic linkage is that selection with one antibiotic automatically selects for multidrug-resistant bacteria. Coselection can influence the spread of antibiotic resistance at the population level by delinking the frequency of resistance to a particular antibiotic from the volume of use of that antibiotic (**Figure 3**). Coselection could also protect a resistance determinant with a significant fitness cost from the purifying selection that would otherwise act to reduce its frequency in the population. There are several reported examples, most likely explained by coselection, where discontinuation in the clinical use of a particular class of antibiotics was not accompanied with the hoped-for decrease in resistance frequency in the community (13, 39, 112). In principle, coselection could also work in the opposite direction, to increase the frequency of a drug-susceptible allele, leading to clonal replacement throughout a population.

The examples given above primarily involve situations in which resistance to antibiotics is acquired by HGT. However, coselection can also occur at the level of the organism, and in

this context globally successful bacterial clones are important examples of coselection. These are antibiotic-resistant clones of important pathogens—for example, *E. coli* ST131 (77); *K. pneumoniae* ST258 (27, 28); *Salmonella enterica* DT104 (67); and *P. aeruginosa* ST146, ST235, and ST111 (86)—that owe their global predominance to having a genotype that successfully combines antibiotic resistance, virulence factors, and transmission features, into a high-fitness biological package. In these successful clones the coselection of genetic factors affecting virulence and transmission, together with resistance determinants, is probably a major determinant of their global success. Recent studies are casting light on the origins of some of these clones and suggesting possible reasons for their successful global expansion. The history of the multidrug-resistant *E. coli* ST131 clone has been mapped using whole-genome sequence analysis of current and historical isolates (77). The evidence suggests that within the B2 phylogenetic group of *E. coli*, resistance to fluoroquinolones evolved by mutation in a strain carrying the *fimH30* allele (a variant of a type I fimbrial adhesion gene) (58, 94). A plasmid carrying CTX-M-15 became fixed within this lineage, and this clone then became globally prevalent by clonal expansion (11). Almost all ST131 strains are multidrug resistant, with their resistance profile including trimethoprim-sulfamethoxazole, amoxicillin, amoxicillin/clavulanic acid, fluoroquinolones, and extended-spectrum cephalosporins (31, 81). Why this particular clone of *E. coli* is so successful is still unclear, but the clone is associated with a high level of transmissibility in both household and hospital settings (50, 57, 77). The evolutionary trajectory of ST131 outlined above is essentially linear. In contrast, the globally successful *K. pneumoniae* clone ST258 involved the creation of a mosaic genome by a major chromosomal recombination event between two different *Klebsiella* sequence types, ST11 and ST442, together with the acquisition of an integrative conjugative element, ICEKp258.2, and several smaller chromosomal alterations (28). ST258 clones are specifically associated with narrow-host-range IncF plasmids carrying KPC genes (27). Sequence analysis of ST258 clones has revealed that they carry resistance genes covering all major classes of antibiotic (66, 117, 118) and also have reduced expression of porins, leading to colistin resistance (30). Given the existence of globally successful multidrug-resistant clones that must, by definition, combine favorable virulence and transmission characteristics, selection of resistance with any of the relevant antibiotics will promote the maintenance of a high global frequency of resistance to each of the colinked antibiotics carried by these successful clonal types.

In summary, linkage between genes that each respond positively to a different selection pressure (e.g., antibiotics, virulence features, transmission) will increase the probability that all of these linked genes together reach a high frequency in a population (**Figure 3**). Accordingly, coselection combines two important evolutionary strategies for success: (*a*) having more tickets increases the chance of winning the genetic lottery, and (*b*) being a member of a successful team increases the chance of winning. In reality, pathogens are likely to be exposed to multiple antibiotics, simultaneously or successively, placing a very high selective value on organisms that are multidrug resistant. By definition, genetic linkage implies that the relative fitness of any individual gene will be selectively evaluated by evolution in terms of its genetic context. In principle, according to the effect of Muller's ratchet, an entire linkage group could be driven to extinction by the accumulation of detrimental mutations (5), but in bacterial populations this effect is countered by the effects of occasional recombination by HGT. Coselection has implications for the evolutionary trajectories of antibiotic resistance determinants. The most prevalent genes/mutations in clinical isolates may not necessarily be the best in the sense of conferring the highest level of resistance at the lowest fitness cost, but they could in many cases be both phenotypically adequate and lucky in terms of their genetic context. Being in the optimal genetic linkage group could, in terms of global genetic success, outweigh the intrinsic fitness value of an individual gene.

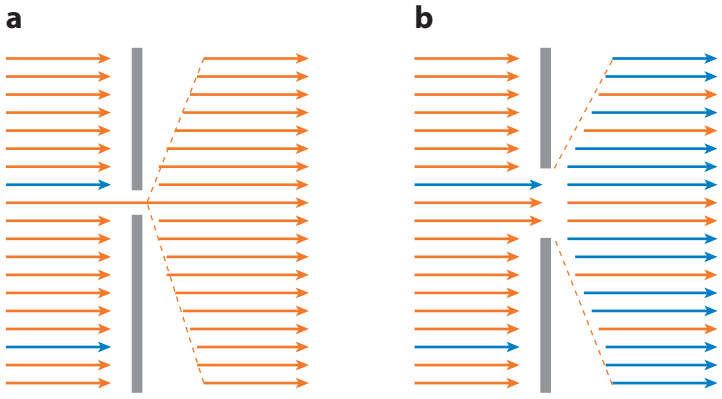

Figure 4

Population bottlenecks affect evolutionary trajectories. Orange arrows represent lineages of high-frequency mutants with relatively low fitness. Blue arrows represent low-frequency mutants with relatively high fitness. (*a*) In a population with a narrow transmission bottleneck (e.g., many infections are initiated by only a few bacteria), the most common mutants (*orange*) are most likely to be transmitted and found in the infecting population, even though they do not have the best phenotype. (*b*) When the transmission bottleneck is wider, both the common orange mutants and the rare blue mutants are transmitted. Because of their higher relative fitness, the blue mutants increase in relative frequency in the population, displacing many or all of the orange mutants.

3.3. Population Bottlenecks Influence Evolutionary Trajectories

In addition to the existence of successful clones there are several other features associated with bacterial pathogens at the population level that can influence the trajectory of antibiotic resistance evolution. Two of the most important are transmission bottlenecks and clonal interference (**Figure 4**).

When a population of bacteria is placed under antibiotic selection, rare resistant mutants within the population will have an advantage over susceptible bacteria. However, for any bacterial population under selection, the probability that a resistant mutant will be selectively enriched depends on several parameters: the mutation rate to generate a resistant mutant, the size of the population under selection (the interplay between these two parameters affects the probability of generating a mutant in that population), the relative fitness of the resistant mutant (which affects the rate of enrichment of that mutant in the population), and the size of the transmitted population responsible for establishing new infections. The mutation rate itself depends on the type of mutation that is required to confer a resistance phenotype, whether it requires a relatively rare amino acid change, a more common gene knockout, or a very common gene amplification (8), as discussed in Section 2.

The multistep evolution of fluoroquinolone resistance in *E. coli* provides a good illustration of how the interplay between mutation rate, population size, and relative fitness influences the trajectory of resistance evolution (54). In this evolution the first mutation to be selected is a single amino acid alteration in DNA gyrase that alters the primary drug target. Resistance mutations in *gyrA* occur at a low rate, as expected (small mutational target size), but there are several different substitution mutations in *gyrA* that can confer resistance. The specific mutation that is most frequently selected in clinical isolates, S83L, confers the biggest increase in MIC with the lowest associated fitness cost (54). Thus, the first step in resistance evolution can be understood in terms of the interplay between resistance and fitness.

The second step in this evolutionary trajectory is more interesting. The primary mutation in *gyrA* increases MIC from approximately 0.016 mg/L to 0.25 mg/L. The maximum MIC associated with a second-step mutation is 0.75 mg/L. Interestingly, there are two very different types of second-step mutations that can confer this level of resistance: Mutations in regulators of the major efflux pump, AcrAB-TolC, occur at the very high rate of $>10^{-6}$ per cell per generation; and amino acid substitution mutations in *parC*, encoding topoisomerase IV, the secondary drug target, occur at the very low rate of $<10^{-9}$ per cell per generation. When second-step mutants are selected by standard in vitro procedures (plating aliquots of culture on selective agar), the overwhelming majority of mutants have mutations in efflux-regulator genes, as expected because of the huge difference in mutational target sizes and mutation rates. However, resistant clinical isolates overwhelmingly carry mutations in *parC* rather than the more frequently occurring mutations in efflux regulator genes (54). The likely explanation is that the *parC* mutations have a smaller fitness cost than mutations that upregulate drug efflux, and clinical isolates are probably under strong selection to maintain fitness. This hypothesis was supported by the outcome of an in vitro selection for second-step resistance mutations made in a nonstandard way (54). When cultures with a primary resistance mutation were evolved for increased resistance, using a transmission bottleneck large enough to allow the occurrence and transfer of both classes of mutations, the evolutionary outcome matched that seen in clinical isolates. Almost all selected mutants carried a mutation in *parC* as their second resistance mutation (54). This suggests that in selecting clinical resistance, low fitness cost is an important parameter and that mutation supply is sufficiently large that rare low-cost *parC* mutations can be selected and fixed in the population in preference to frequent but high-cost efflux regulator mutations.

The message from this example is universal: For a given antibiotic selective pressure, small transmission bottlenecks favor mutations that arise at a high frequency, whereas large transmission bottlenecks favor mutations that confer that lowest fitness costs (**Figure 4**). In this way, population bottlenecks will act to constrain the trajectories of resistance evolution by limiting the mutation supply and thus affecting the degree of competition between alternative mutational solutions. One important caveat is that bacterial mutants with increased mutation rates (mutator bacteria) will generate mutants with the selected phenotype at a higher than normal rate and thus reduce the effect of smaller transmission bottlenecks. This may explain why approximately 1% of clinical and natural isolates of several species, including *E. coli* (35, 65, 85), have a mutator phenotype. Accordingly, when the mutation supply is small (small population size, low mutation rate, or small mutational target size), the first mutation to arise will have a high probability of being selected, enriched, and transmitted. When mutation supply is less limiting (larger population size, higher mutation rate, or larger mutational target size), many different mutations may arise and compete in a population and the mutation with the highest competitive fitness will be selectively enriched and consequently have a higher probability of being transmitted. Thus, transmission bottlenecks, and the interplay between mutation supply and fitness, will significantly influence the trajectory of evolution.

A population of bacteria under antibiotic selection may contain several genetically different antibiotic-resistant mutants simultaneously (75, 76, 111). In such a situation clonal interference (competition between asexual clones) can influence the evolutionary trajectory (**Figure 4**). Thus, different mutants arising independently will compete not just against the susceptible parent but also against each other. This competition will typically lead to a population with one dominant clone (43, 122). When populations are large and/or mutation rates are high, there will be larger numbers of different competing resistance mutations, thereby increasing the potential for clonal interference. Clonal interference has been shown to influence evolutionary trajectories in the adaptation of antibiotic resistance plasmids to a host (53) and in the adaptation of mutant resistant bacteria to the fitness costs of resistance (44).

4. CONCLUSIONS AND PERSPECTIVES

Areas where additional knowledge is urgently needed to predict evolutionary trajectories include (*a*) the rates of resistant mutant formation by HGT, (*b*) the influence of selection pressure, and (*c*) the influence of population structure.

We have a good understanding of mutant formation rates within a genome (point mutations, knockouts, amplifications) but a very poor ability to predict the probability of HGT leading to antibiotic resistance. The potential of the global microbiome as a source of resistance genes is enormous, and genes for most classes of antibiotics have been discovered in the human gut microbiome (109), the soil microbiome (33), and even geographic locations thought unlikely to have ever been exposed to human sources of antibiotics (91). However, what is still lacking is a deep understanding of the factors that influence the probability that any particular gene will transfer into a relevant human pathogen.

Growth competition assays under laboratory conditions may be a very useful tool for correctly predicting clinical evolutionary trajectories (54), but more data are needed to understand the effects of fluctuating selections, and of the complex multifactorial selections likely to be frequently encountered in many natural environment (46).

Mathematical modeling based on experimental data can provide good insight into how the interplay between population sizes, mutant formation rates, and transmission bottlenecks influences the trajectories of clinical resistance evolution (54). However, there is a paucity of data on population structures in relevant environments (clinical and natural) where resistant mutants form and are selectively enriched.

A major challenge in the near future will be to predict the evolution of resistance to novel classes of antibiotics before clinical resistance develops postmarketing. For this to be realistic we must address the gaps in basic knowledge discussed in this review, and in particular the points outlined in the summary statements above (HGT, complex selective environments, relevant population structures).

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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