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Lessons from the Environmental Antibiotic Resistome

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Abstract

Antibiotic resistance is a global public health issue of growing proportions. All antibiotics are susceptible to resistance. The evidence is now clear that the environment is the single largest source and reservoir of resistance. Soil, aquatic, atmospheric, animal-associated, and built ecosystems are home to microbes that harbor antibiotic resistance elements and the means to mobilize them. The diversity and abundance of resistance in the environment is consistent with the ancient origins of antibiotics and a variety of studies support a long natural history of associated resistance. The implications are clear: Understanding the evolution of resistance in the environment, its diversity, and mechanisms is essential to the management of our existing and future antibiotic resources.

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INTRODUCTION

It is instructive to consider how antibiotics have transformed medicine in less than a century. Before the availability of the first broadly useful antibiotics in the mid-1930s, life expectancy was much shorter and infectious and related diseases were the dominant causes of death. With the advent of antibiotics, these statistics dramatically reversed (4). For the first time in our history, humans were more likely to die from chronic diseases of old age (e.g., cancer and cardiovascular disease) than from infection. Preventative approaches to improve public health and access to vaccines also played a significant role in the pivot away from infection as a leading cause of death. However, antibiotics remain the most effective method to deal with acute infections. Their availability enables clinicians to engage in procedures unthinkable without control of infection. The list of these advancements is now well known; antibiotics support all major surgeries, cancer chemotherapy, organ transplantation, and a myriad of other common medical procedures. Antibiotics are cornerstones of modern medicine.

What is also well known is that microbial resistance to antibiotics is a correcting force of such magnitude as to place at risk the advances of past decades (8, 110). Resistance has been linked to antibiotic use since the 1930s. The steady supply of new antibiotic scaffolds in the golden era of antibiotic discovery followed by new varieties of these scaffolds designed to circumvent resistance in the era of antibiotic medical chemistry that followed served to allay concerns of resistance outstripping antibiotic efficacy (22). This sanguine view is now outdated. The last new antibiotic scaffolds to be successfully introduced as drugs were first reported over 30 years ago. The productive medicinal chemical elaboration of some of our most successful classes of antibiotics, such as the β-lactams and the fluoroquinolones, has reached asymptotic levels. All the while, resistance to all antibiotic classes continues to emerge and be transmitted through bacterial communities and across the globe. We are now in the era of resistance (22).

Perhaps the main reason pundits are increasingly asking whether we are about to enter a postantibiotic age is that we have failed to understand antibiotic resistance as the product of evolution, occurring in all ecological niches and over geologic time periods. The industrialization of antibiotic production and their extravagant use in humans and animals over past decades have

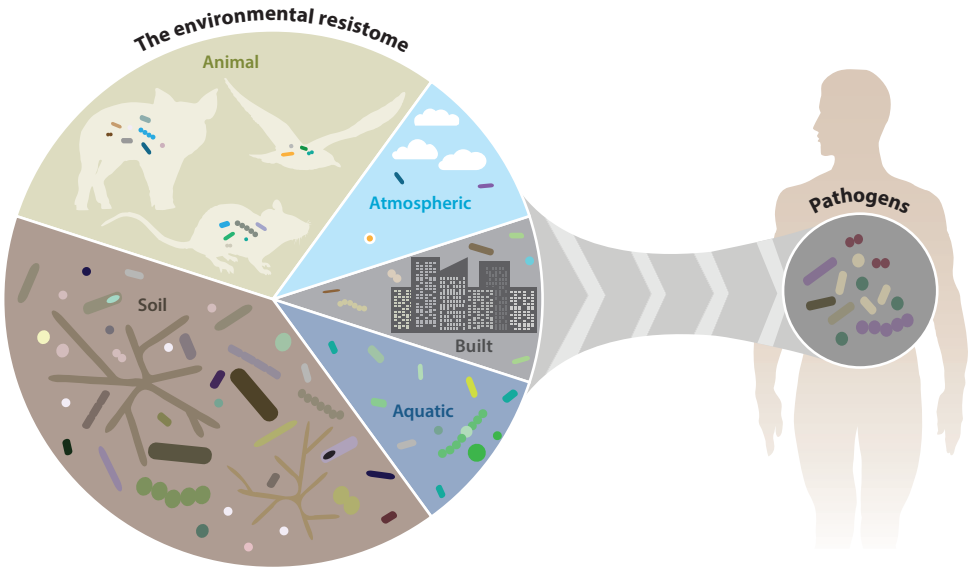

Figure 1

Resistance is readily detected in soil, aquatic, atmospheric, and built environments. Soils are likely the major reservoir of resistance genes and source of their diversity, given the density of microbial species there. Wild and domestic animals are also sources of resistance genes and microbial species that are shared with humans, including many that cause disease. The selection and mobilization of resistance elements coincident with antibiotic use results in gene flow from the environment to the pathogens that cause disease.

provided the global selective pressure, never experienced by microbes on the planet at this scale, for the amplification, diversification, and dissemination of resistance. For example, industrial effluent from antibiotic production sites discharged into the environment can reach levels 1,000 times higher than concentrations typically used to kill bacteria (80). Our failure to understand resistance through the lens of evolution and ecology is a barrier that must be overcome to avoid a postantibiotic era. A turning point in this new view of resistance is the increasing realization over the last decade that antibiotic resistance in the clinic mirrors and in many cases has its origins in environmental microbes (**Figure 1**) (45, 53, 155).

The environment, with its vastly larger numbers of bacteria than those that cause disease and its intimate association with antibiotic production by microbes, has been mostly ignored over the course of the antibiotic era. Only now are we realizing the critical role of environmental microbes as wellsprings of resistance elements. By understanding the natural history of resistance in the environment and the evolutionary forces that shape and affect antibiotic action and resistance, we can begin to view antibiotics through an ecological lens and use this knowledge to shape a new postresistance era.

THE ANTIBIOTIC RESISTOME

The antibiotic resistome is a framework in which to study resistance beyond the narrow point of view of the clinic, taking a more expansive perspective to encompass resistance in all its forms, including in noninfectious environmental microbes (117, 154). The genetic and functional diversity in the resistome is vast and reflects the billions (10^9) of years of evolution of microbes in close contact with toxic molecules of many origins. These include inorganic metals (9, 83) and

organic compounds made by microbes, plants, and animals (17). The molecules we term antibiotics are a subset of these toxic compounds (and here we must also include human-made compounds, as they are subject to the same evolutionary forces as natural substances). In many cases, the molecular mechanisms that have evolved to evade nonspecific toxic molecules are identical and readily repurposed for antibiotic resistance.

Analogous to the complex immune systems of metazoans that have both innate and adaptive components, the resistome includes intrinsic and acquired mechanisms (**Figure 2**). Intrinsic

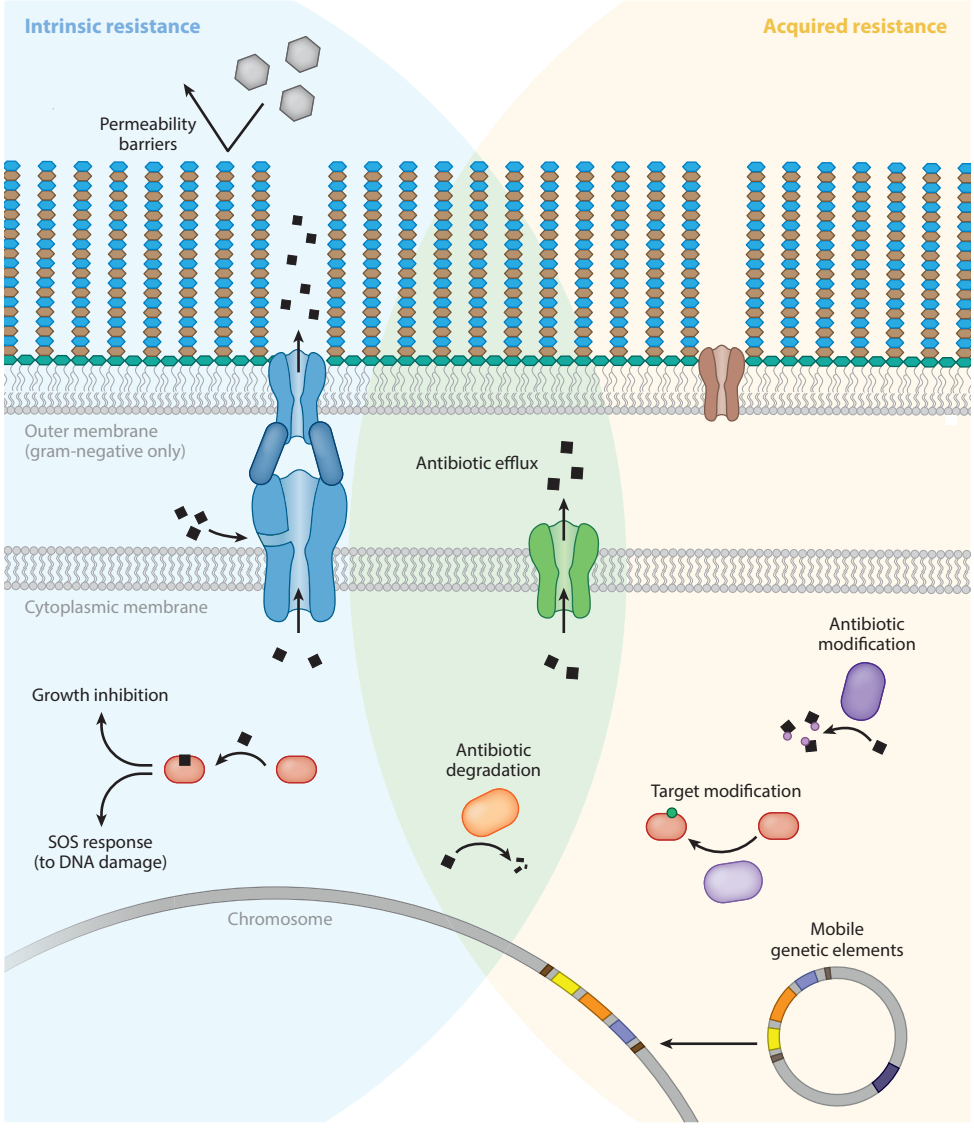

Figure 2

The intrinsic and acquired antibiotic resistomes. Bacteria can be resistant to antibiotics via intrinsic mechanisms, including drug permeability, efflux, degradation, and upregulation of genomic mutation. Acquired resistance includes altered targets, drug inactivation, and efflux, most often acquired through the horizontal transfer of resistance elements from other species and genera.

resistance includes mechanisms that have evolved as a general response to toxic molecules; these include the SOS response that relaxes DNA replication fidelity (30), broad-spectrum efflux pumps (84, 95), chromosomally encoded inactivating enzymes such as β-lactamases (72), and barriers to entry such as porins and the outer membrane of gram-negative bacteria (108). Acquired resistance comprises mechanisms that evolve as countermeasures to particular antibiotics or scaffolds, often through horizontal gene transfer (HGT). These include compound-specific efflux pumps, expression of non-sensitive targets, and enzymes that modify targets or the antibiotic molecules. The vast array of mechanisms that act to block antibiotic action and the astonishing number of bacterial species on the planet [estimated to be 10^{12} species (87) from 96 phyla (68)] conspire to make the resistome a formidable obstacle to antibiotic drug discovery.

The sequencing of microbial genomes from diverse phyla and environments reveals that most (perhaps all) bacterial genomes harbor resistance elements, many in the form of intrinsic mechanisms (28, 43). These are the scars of the natural history of bacteria and the diversity of toxic molecules that they have encountered, including antibiotics. Furthermore, many of these genomes display a variety of HGT signatures in the form of genes and pseudogenes encoding integrases, transposases, and gene sequences that confirm a long history of gene mobilization within and across microbial species and genera (135). Such genomic resistance islands are found in many bacterial genera, often associated with integrons, and can accumulate dozens of resistance genes (50, 54, 60, 61, 99). The mycelial structure of many soil microbes may greatly facilitate HGT and the exchange of antibiotic resistance elements (16). Mobile elements (e.g., plasmids and transposons) are found in many bacteria and offer facile routes of HGT that often do not respect species or genus boundaries. As a result, antibiotic resistance elements have diversified and moved across environments over millennia.

Analysis of the resistance gene makeup of environmental microbes in comparison to antibiotic naive pathogens [e.g., from compendia of bacteria from the preantibiotic era, such as the Murray collection (7)] clearly shows that resistance elements are highly enriched in the former and rarer in the latter. Waksman (146) was among the first to note the antagonistic interactions between environmental microbes (bacteria and fungi). A more systematic study of the resistome of ~500 actinomycetes collected from a variety of soils revealed that these bacteria are resistant to, on average, 7–8 antibiotics out of 21 tested at 20 μg/mL (40). In contrast, a survey of preantibiotic-era *Salmonella* strains identified numerous plasmids but no resistance genes (75). A retrospective review of hospital- and community-acquired pathogens (e.g., *Streptococcus*, *Staphylococcus*, *Neisseria*, *Salmonella*, and *Haemophilus* species) marked the steady rise and spread of resistance from 1935, when most strains were highly antibiotic sensitive, to 1975, when resistance was common (44). The differences in antibiotic resistance before 1940 between pathogens and environmental microbes reflect the more challenging and diverse chemical ecology experienced by environmental bacteria in comparison to pathogens, many of which are commensal.

Resistance genes present in bacterial genomes may not be expressed [silent resistance (116)], and as a result resistance genotype may not correlate with phenotype as measured by standard minimal inhibitory concentration (MIC) in the laboratory. For this review we consider antibiotic resistance to be driven by genotype; i.e., a bona fide resistance gene is one that confers protection from the antibiotic (increase in MIC) when expressed. We do not consider mechanisms of antibiotic tolerance such as persistence, biofilm formation, and stochastic gene amplification, though these do contribute significantly to the global challenge of antibiotic resistance.

THE ANCIENT RESISTOME

The prevalence of intrinsic and acquired resistance mechanisms in virtually all sequenced bacteria is evidence of the profound impact of antibiotics and other toxic substances on the course of

microbial evolution (115). Establishing precisely when antibiotics arose during microbial evolution is difficult. Using a molecular clock approach on a small sample of antibiotic biosynthetic gene clusters, Baltz (10) suggested that antibiotic scaffolds appeared tens (daptomycin, vancomycin) to hundreds (erythromycin, streptomycin) of millions of years ago. A similar investigation of β-lactam antibiotic biosynthesis, a capacity that is shared by fungi and bacteria, suggested that transfer of the isopenicillin N synthase gene from bacteria to fungi occurred at the time of divergence of gram-positive and gram-negative bacteria, roughly one billion years ago (79). Resistance to antibiotics must have coevolved with antibiotic biosynthesis within producing organisms, and therefore is equally old. Analyses of β-lactamase gene divergence by Hall and Barlow set the emergence of metallo β-lactamases roughly one billion years ago and mobilization of Ser β-lactamases into plasmids tens to hundreds of millions of years ago (11, 58, 59). These in silico studies of gene divergence are challenging, based on many assumptions including the constant rate of genetic change, which we know to be inaccurate, but they all point to the fact that antibiotic biosynthesis and resistance have evolved along with bacterial radiation for millions to billions of years. They are unquestionably ancient molecules and part of the fabric of microbial ecology and evolution.

While these gene divergence studies are informative, they do not offer concrete evidence of the ancient origins of resistance. The reasons for this are obvious: Antibiotics, DNA, and proteins degrade over time and are lost from the fossil record. Nevertheless, there have been efforts to explore ancient DNA samples for the presence of resistance. Such studies require access to well-preserved, ancient DNA—difficult in most environments where preservation is not supported by ecological conditions (65). The permafrost of subpolar regions does offer a unique environment for the collection of DNA and even viable cells that are tens of thousands of years old (73). Renowned for their caches of exquisitely preserved extinct mammals, the Arctic permafrost regions offer an opportunity to sample DNA that predates the modern antibiotic era by millennia. A metagenome analysis of 30,000-year-old Yukon permafrost yielded DNA from extinct megafauna, such as mammoth, in addition to resistance elements for β-lactam, tetracycline, and glycopeptide antibiotics (39). This study reported the amplification of a complete *vanA* vancomycin resistance gene, overexpression of the protein, determination of the steady-state kinetics of D-alanine-D-lactate formation (an essential biochemical step in vancomycin resistance), and solution of the 3-D structure of the enzyme. In all ways, 30,000-year-old VanA behaves identically to the modern version that confers resistance in enterococci in hospitals, demonstrating concretely that antibiotic resistance predates human use by millennia.

Using a functional metagenomic approach, where a gene library is constructed from a metagenome (in this case, 5,000- to 6,000-year-old Nunavut permafrost) and selected for antibiotic resistance phenotypes, a recent study identified several aminoglycoside, penicillin, and tetracycline resistance elements (114). Other studies of living bacteria recovered from permafrost samples from Siberia and Antarctica have also reported resistance elements and even resistance genes on mobile plasmids (101).

Additional evidence for ancient resistance elements is emerging from other well-preserved samples. For example, dental plaque from human skeletons from medieval Germany dating to 950–1200 CE reveals oral metagenomes rife with known efflux genes, β-lactamases, and other intrinsic antibiotic resistance elements (149). Sampling of the intestinal contents of a 1,600-year-old Peruvian mummy identified a similar diversity of antibiotic resistance genes (130). These ancient DNA studies conclusively demonstrate the presence of antibiotic resistance genes in bacteria long before the industrialization of antibiotic production in the 20th century.

Contemporary samples of "antibiotic-free" environments further support the prevalence of resistance in microbial genomes unrelated to modern human use. With the advent of globalization, it is difficult to identify regions of the planet that are clearly unaffected by modern humans—a fact

that has been interpreted as the hallmark of a new epoch, the Anthropocene (160). Traces of human activity are found in the distribution of plastics, pollutants, and other markers of industrialization across the globe: from the polar regions to the highest mountain ranges and the depths of the oceans. However, environments untouched by humans for millennia are found beneath the surface of the planet. The Lechuguilla Cave system is one such location. Having been sealed from the surface for millions of years, it was first entered in 1986. The microbes that live on the cave walls form a biofilm that is alive but has been separated from surface organisms for approximately four million years. A sampling of the cultured microbiome of the cave revealed both gram-positive and gram-negative bacteria that were highly resistant in a survey of 26 different antibiotics at 20 μg/mL (18). Investigation of the genetic and biochemical details of one of these strains, *Paenibacillus* LC231 resistant to 26 of 40 tested antibiotics, reveals members of known resistance mechanisms widespread in clinical isolates (β-lactamases, aminoglycoside-modifying enzymes, etc.) in addition to several new mechanisms, including a ribosome methyltransferase conferring clindamycin resistance, a bacitracin amidohydrolase, and a capreomycin acetyltransferase (113). Similarly, the microbiomes of uncontacted American Indians also contain numerous intrinsic antibiotic resistance genes, akin to the microbiomes of industrialized comparator populations (26). These studies of genomes and metagenomes of modern but antibiotic naive environments reveal the deep embedding of resistance elements in microbial genomes, reflecting a long relationship with antimicrobial substances.

It is important to acknowledge that while antibiotic resistance is indeed easy to identify in ancient and antibiotic naive modern samples, the accumulation of multiple antibiotic resistance elements through HGT, coupled with increased rate and scope of movement across species and genera into pathogens, is closely linked to human use of antibiotics occurring since the mid–20th century. This is borne out by the examination of the resistance gene makeup of ancient bacterial pathogens such as *Yersinia pestis*, responsible for the Black Death (14th century) (21) and the Justinian plague (6th to 8th centuries) (145), and *Vibrio cholera* (cause of an 1849 cholera outbreak in Philadelphia) (35), none of which have resistance genes other than those expected in the intrinsic resistome of these microbes.

THE ENVIRONMENTAL RESISTOME

The Oxford English Dictionary defines environment as "the surroundings or conditions in which a person, animal or plant lives or operates." Since microbes occupy the entire planet, the relevant locales include soils, water, air, and built environments. Recent metagenomic studies of contemporary samples across all of these environments demonstrate the dramatic depth and breadth of resistance genes outside of clinical settings (46, 107, 112).

Soils

Soils are "the most complicated biomaterial on the planet" (159). They are highly variable, dependent on water content, mineral composition, oxygen concentration, and nutrient availability. The result is a highly diverse ecosystem that can change over time (e.g., because of seasonal variation) and distance, ranging from microns to kilometers. The denizens of soils include microbes (bacteria, fungi, archaea, cyanobacteria, protozoans, phage, and other viruses), plants, and larger animals, including nematodes, arthropods, worms, and burrowing mammals. Waksman (147) expounded on the complexity of soil microbe communities over 70 years ago. He summarized the interactions between microbes as associative (symbiosis, growth promotion, the liberation of nutrients from complex forms, consumption of oxygen), competitive (for nutrients or space), or antagonistic

(production of growth-inhibitory substances). The latter can be passive, e.g., change in the pH of the local medium, exhaustion of nutrients, or active, in the form of excretion of toxic compounds, pigments, or lytic enzymes. Using culture-based approaches, Waksman and others in the early 20th century recognized that many microbial species produced selectively toxic metabolites. This realization not only launched the golden era of antibiotic discovery but also identified some of the first anticancer and antifungal agents as well as multiple other drug classes—from immune suppressants to cholesterol-lowering agents. Soils indeed offered a wealth of leads for new medicines and mining of soil microbes for such compounds dominated efforts in the pharmaceutical field for decades.

Bioactive compounds from soil microbes are a boon to drug discovery but are rarely investigated for their intrinsic roles in producers or their impact on the ecology of the soil. In fact, the thousands of such compounds reported in the literature (17) represent only a small part of the chemical space available to these organisms. Sequencing of the genomes of soil microbes, including bacteria of the genus *Streptomyces* and other filamentous actinomycetes, reveals that they have the genetic capacity to produce a dizzying spectrum of compounds. Each actinomycete genome has on average 20 to >30 genetic programs encoding bioactive compounds, many of which are antibiotics (77). Filamentous fungi are similarly prolific producers of secondary metabolites, with approximately 40 to 80 biosynthetic gene clusters per *Aspergillus* genome, for example (70). The precise roles of most of these compounds in soil environments are rarely known with confidence (109). Certainly many do have antimicrobial activity, but whether this is their primary role is disputed (33, 157, 158). The killing activity of antibiotics is concentration dependent, and sub-MIC concentrations can have pleiotropic effects on bacterial gene expression and metabolite production (102, 136, 142). The fact that antibiotic quantities in soils are difficult to measure, and likely generally much lower than concentrations that can be secreted in the lab, fuels doubt that cell-killing is their primary activity.

Imaging mass spectrometry experiments demonstrate that production of secondary metabolites, including antibiotics, is highly dependent on growth conditions and proximity to other microbes (104, 141, 150). Not surprisingly, what is evident from these studies is that there is a gradient where antibiotic concentrations are high close to the producing cells diminishing as compounds diffuse out into the medium. Furthermore, it is well known to researchers purifying antibiotics and other secondary metabolites that these are very frequently physically associated with producing cells. Microbes live on the micron scale, so although antibiotics may have multiple effects on adjacent cell metabolism and gene expression not directly related to cell death, as proximity of cells increases, antibiotics very likely do have antibiotic activity. Supporting evidence for this hypothesis comes from the genetically diverse and extensive soil resistome evolved to attenuate antibiotic killing activity.

The similarity of the molecular mechanisms of resistance observed in the clinic to those in soil bacteria, in particular, the self-protection mechanisms of antibiotic-producing bacteria, had been made over the past four decades (15, 31, 94). These studies tended to be ad hoc. The first systematic effort to explore the soil resistome revealed that antibiotic resistance in spore-forming bacteria was ubiquitous and diverse (40). The mechanisms of resistance included those equivalent to those circulating in clinical isolates and also strategies not known in pathogens.

In another study that explored a broader spectrum of bacterial genera, 412 strains isolated from a variety of soils were tested for resistance against a panel of 24 drugs (148). The majority (80%) were multidrug resistant, confirming the diversity and frequency of resistance in soils. Efflux mechanisms were the primary source of the multidrug phenotype, but drug inactivation was also prevalent, in particular for penicillin resistance. Common β-lactamases circulating in the clinic were not detected, pointing to the importance of the intrinsic β-lactamases.

The capacity of soil bacteria to inactivate antibiotics extends even to the extreme of subsistence, as some strains can use antibiotics as their sole carbon source (32). These bacteria span a range of genera, and this ability is not confined to a small group of organisms, nor is it limited to specific classes of antibiotics, as natural products and synthetic drugs were both capable of supporting growth of bacteria. This spectrum speaks to the remarkable resilience and diversity of soil bacteria to detoxify a myriad of compounds, reflecting the ancient lineage of these organisms.

Using unbiased functional metagenomic strategies, several researchers have identified novel antibiotic resistance elements in a number of soils (2, 46, 114, 123, 140). These studies reveal a great diversity of β-lactamases, and aminoglycoside-modifying enzymes in particular. Genetic diversity of resistance is not limited to these antibiotic classes, however, and other studies reveal an extensive collection of tetracycline-inactivating enzymes, for example (48). In general, the soil resistance elements are similar but not identical to those circulating in human pathogens. However, this is not always the case (49) and likely reflects the limits of these surveys and the relative abundance and diversity of resistance in soil. Furthermore, they are often associated with mobile genetic elements (90).

Human activity, in particular, agricultural practices such as application of manure and sewage sludge, can significantly alter the composition of soil resistomes. Several studies have shown that such practices enrich soil with genes that are often found in pathogens, thereby substantially mixing human and environmental resistomes (25, 64, 107, 151, 153, 156).

Aquatic Environments

Rivers, lakes, and marine systems are also reservoirs for resistance (reviewed in 162). These environments have lower overall bacterial densities in comparison to soils, but their sediments are rich in diverse bacterial genera. Several studies have noted the resistance burden in aquatic environments. The proximity to human activity, in particular, pharmaceutical and other polluting industries, dramatically increases the resistance load in aquatic environments. Larsson and coworkers have demonstrated that such environments are highly contaminated with antibiotics (80) and that associated sediments are rife with resistance elements and multidrug-resistant bacteria at levels much higher than at pristine locations (13, 47, 78, 112, 127). These results are supported by several other studies that link human impact on aquatic systems with increases in resistance across the globe (46, 107, 161), including some of the most concerning resistance elements, such as the NDM-1 carbapenemase (91), and vancomycin resistance in staphylococcal isolates (69).

While human activity correlates with high levels of antibiotic resistance, these genes are detected in even low-impact environments, such as glacial ice (143) and Red Sea brine pools (41). Suspended sediments (flocs) have also been sampled for resistance elements in pristine, agricultural, and urban lakes, and all were shown to harbor antibiotic resistance (38). A survey of seawater samples also demonstrated the widespread presence of resistance elements (63). Like soils, aquatic environments are reservoirs of resistance organisms and genes.

Atmospheric Environments

Bacteria become airborne in particles of dust and other aerosols and can travel across continents (29). Exploration of atmospheric samples identifies a broad diversity of microbes (5). The resistance burden in these samples is detectable but low (3, 98). In antibiotic-intense environments, such as farms, this is not the case, and antibiotic resistance genes are readily identified in air samples (51, 52). Smog in highly urbanized areas such as Beijing has also been shown to carry a diverse set of antibiotic resistance elements (112). These results reveal the potential of airborne antibiotic

resistance elements to become a health problem. Dust from natural disasters or storms is associated with outbreaks of fungal infections (12), and it is conceivable that antibiotic-resistant bacteria or their genes could increasingly be a problem.

Built Environments

Exploring the microbiomes of built environments is an area of active research (82, 103). Measuring the associated antibiotic resistance diversity is increasingly a component of these studies but is not yet routine. A study of the microbiome of the Boston transit system identified a wide variety of microbes, but a limited number of resistance elements (66). Where there is a strong correlation with the built microbiome and antibiotic resistance is in hospital settings (reviewed in 81, 103). Drug-resistant bacteria are widespread in such environments, including on hard surfaces and linens and in air supplies. Neonatal intensive care units are particularly vulnerable to colonization by pathogens, with associated increased risk of infection.

ANTIBIOTIC RESISTANCE IN ANIMALS

The interplay between the environment, pathogenic microbes, humans, and other members of the animal kingdom shapes the history of infectious disease. Animals are crucial in the spread and evolution of pathogenic bacteria; it should, therefore, come as no surprise that they have a part to play in the dissemination and development of antibiotic resistance. The number of bacterial cells living on and inside humans is roughly equal to the number of our cells (131). While this ratio may fluctuate across the animal kingdom, almost all metazoans possess an associated community of bacteria. Even microscopic animals, such as *Caenorhabditis elegans* (36) and sea sponges of the phyla *Porifera* (138), have defined microbiomes. These microbiomes contain diverse resistance elements, most often in the form of their intrinsic resistomes.

Large-scale animal husbandry facilities, which often employ staggering amounts of antimicrobials for growth promotion and infection control, are environments that are exquisitely poised for the accumulation and spread of mobile resistance elements. Globally, most antibiotics are used on livestock, resulting in a massive expansion and mobilization of resistance in the bacteria that reside on and in these animals (24, 74). The expanding aquaculture industry relies on antibiotics to sustain fish populations existing at far higher densities than would be possible in the wild, and these communities are often ravaged by bacterial pathogens (23). An in-depth treatment of the topic of antibiotics in agriculture is beyond the scope of this review but is intimately linked to the challenge of environmental drug resistance. Furthermore, the microbiota of wild animals is increasingly recognized as a reservoir of resistance genes and zoonotic pathogens. For instance, enteric bacteria resistant to clinically utilized antibiotics have been isolated from rodents, nonhuman primates, foxes, wolves, wild boar, deer, insects, and birds (1, 27, 57, 100, 126). In many cases, these include pathogens with highly concerning resistance phenotypes, such as extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* (132), vancomycin-resistant *Enterococcus* spp. (VRE) (92), and methicillin-resistant *Staphylococcus aureus* (MRSA) (88).

There is even evidence that benevolent human activity can contribute to the spread of resistance into wild animals. Power et al. (121) followed the release of antibiotic resistance into the wild through a breeding program designed to increase wild populations of the endangered brush-tailed rock wallaby. At no point during this program were the captive wallabies exposed to antibiotics. Using culture-independent methods to search for the presence of *int1* in stool samples from the wallabies, this sentinel gene of HGT (55) was present in half of the captive wallabies but was undetected in wild wallabies despite sampling of twice as many animals from multiple

wild populations. Six different integron cassettes were characterized carrying resistance genes to streptomycin, spectinomycin, trimethoprim, and quaternary ammonium compounds. Resistance cassette diversity suggests that these animals acquired *int1*$^{+}$ bacteria on multiple, independent occasions during captivity.

Birds

Wild birds are prodigious carriers of antibiotic-resistant bacteria. Of particular note are migratory birds, which have the potential to disseminate resistant bacteria over their entire geographic range. A wide variety of avian species, including birds of prey, pigeons, songbirds, and many more species, carry drug-resistant enteric bacteria (27, 71, 122). However, certain populations are especially worrisome. For instance, 30% of *E. coli* isolates from black-headed gulls, a European migratory species, were resistant to at least one antibiotic (usually ampicillin or tetracycline) and approximately 10% of resistant strains contain a class 1 integron, suggesting that these resistance genes are mobile (37). Feces from seagulls on the Portuguese coast yielded four distinct CTX-M β-lactamases from 45 cefotaxime-resistant *E. coli* strains belonging to diverse genotypes (132). The presence of CTX-M β-lactamase-positive *E. coli* from gulls was also reported in France (19) and Sweden (20). ESBLs were recovered in *E. coli* from both gull and pelican feces on the Florida coast (119). ESBL (CTX-M and TEM)-positive strains of *E. coli* have also been isolated from seagulls at a nature reserve in Portugal, far from regular human or antibiotic contact (118). Gulls were among the birds sampled at three highly remote arctic locations and shown to carry resistant *E. coli* (133). Collectively these studies highlight the capacity of gull species for the spread of antibiotic-resistant bacteria and raise public health concerns about beaches and other environments where humans may come into contact with gull feces.

A Dutch study of over 400 wild bird carcasses reported that recovery of ESBL or AmpC-positive *E. coli* was significantly enriched in species that were commonly found in and around water (144). Ribotyping of *E. coli* isolated from gulls has been found to be similar to isolates from wastewater and landfills (106), suggesting that resistant strains are moving into wild bird populations through contaminated water. Consistent with this hypothesis, waterfowl, including ducks (85, 144) and Canada geese (100), have been shown to be another significant reservoir of antibiotic resistance. While these studies are informative, the targeted isolation of *E. coli* does lead to a sampling bias of the resistome of wild avian microbiota. In 2011, a functional metagenomic study using DNA isolated directly from gull feces (collected in Maryland, United States) found a significant diversity of resistance genes to penicillins and tetracycline (96). This work identified previously uncharacterized genes conferring resistance to β-lactams (38%) in addition to known class A and C β-lactamases, efflux proteins, and a carboxypeptidase (likely a resistant PBP1a) and six known genes conferring tetracycline resistance. The prevalence of antibiotic-resistant enteric bacteria in wild birds, which should be under minimal selective pressure of direct antibiotic exposure, is concerning.

Insects

Insects are members of one of the most successful animal taxa. They are remarkably diverse and are ubiquitous in terrestrial ecosystems. Insects have both the ability to transmit resistant bacteria, and their own distinct microbiome-associated resistomes. Oil flies (*Helaeomyia petrolei*) inhabit the asphalt seeps of Rancho La Brea in Los Angeles, California, known colloquially as the La Brea tar pits. The larvae of these flies are laid directly into the oil of La Brea, and their guts have been shown to contain asphalt, tar, and petroleum. Kadavy et al. (76) cultured bacteria from

the gut of oil fly larvae, including nine *Providencia rettgeri* isolates. All were resistant to multiple antibiotics, including tetracycline, nitrofurantoin, and polymyxins, and many were able to tolerate high concentrations of organic solvents. In some of these strains, this tolerance was potentiated by tetracycline implicating inducible efflux as the mechanism for the multidrug resistance.

Honey bees (*Apis mellifera*) contain a well-defined gut microbiota in which eight bacterial species make up around 99% of the community (42). Oxytetracycline has been used for over 50 years in the United States to control hive pests in beekeeping, but in many other countries (Switzerland, Czech Republic, New Zealand) this drug has never been used. Tian et al. (139) used functional metagenomics to identify the tetracycline resistome of the honey bee gut and then used quantitative PCR to monitor levels of these genes across different colonies. Several tetracycline resistance genes, *tetB*, *tetC*, and *tetW*, seemed to be ubiquitous, regardless of colony origin, while genes isolated only from American colonies included *tetD*, *tetH*, *tetY*, *tetM*, and *tetL*. In contrast, wild bumble bees in the United States had not acquired these five genes but still possessed *tetB*, *tetC*, and *tetW*, like the European colonies. The reason for the prevalence of these three genes, even in antibiotic naive populations, is unknown. Using quantitative PCR, the authors revealed a clear association between antibiotic exposure and abundance of tetracycline-resistance genes. These resistance determinants were most abundant in the recently established American colonies, whereas the colonies that had not been exposed to tetracycline in the last two years had markedly fewer copies of these genes; foreign colonies had still lower abundance. The tetracycline resistance genes have the hallmarks of recent HGT (mobile genetic elements, high identity), including *tetBCW*, with some sequences being identical to those of human pathogens.

In another exploration of insect-associated microbes and resistance, isolates from the gypsy moth (*Lymantria dispar*) gut were explored using functional metagenomics (1). This study identified three novel resistance genes, an ESBL, an RND (resistance–nodulation–cell division) family efflux pump, and a transcriptional activator of efflux. In another study, VRE and MRSA were cultured from bed bugs (*Cimex lectularius*) found on patients infested with the parasites (89), demonstrating the capacity of insects to harbor pathogens and resistance elements.

It has been hypothesized that insects play a central role in the transfer of antibiotic-resistant bacteria from agricultural settings to other environments (163). For instance, Literak et al. found that flies (*Musca domestica*) associated with a swine farm were hosts to identical strains of resistant *E. coli* recovered from the swine themselves (86). The same phenomenon has been reported for dairy farms (129). These studies, coupled with the fact that flies can travel many kilometers from a source, suggest insects can rapidly spread these bacteria, and their associated resistance genes, into other environments (105, 152).

Anthropogenic Effects on Resistance in Animal Populations

Studies of resistance in wild animal populations tend to focus on *Enterobacteriaceae* such as *E. coli* because of their tractable nature, relevance to human disease, and use as a marker of anthropogenic pollution. An influential 1999 study by Gilliver et al. (57) reported widespread antibiotic resistance (β-lactams, tetracycline, and trimethoprim) in coliforms isolated from wild rodents in northern England over a three-year period. These animals should not have been exposed to antibiotics and presumably had minimal contact with animals that had, meaning that these resistance determinants were present in the absence of direct selection (57). In contrast, researchers conducted a similar study on ungulates and rodents in western Finland. *Enterobacteriaceae* recovered from these animals showed minimal resistance (2/186 isolates) to 12 clinically relevant antibiotics (111). The discrepancies between the two studies may reflect a combination of ecological factors including human population density; less intensive animal agriculture; a 1996 ban on antibiotics in animal

feed in Finland; and the long, cold winters, which hinder the spread of enteric bacteria within populations. Similarly, a study of *Enterobacteriaceae* from iguanas native to the Galapagos Islands with little human contact revealed an almost complete absence of acquired resistance elements (137).

There is growing evidence that ties human exposure to antibiotic resistance in wild populations. Skurnik et al. (134) isolated *E. coli* from six animal populations representing increasing degrees of human exposure: animals from Gabon, the Antarctic, the Pyrenees mountain range, and a forest located near Paris; farm-reared animals; and pets. Beginning with no resistance in the Antarctic- and Gabon-sourced isolates, resistance steadily increased with increased human exposure (134). Nonhuman primates living near people have also been shown on multiple occasions to harbor more resistant *E. coli* isolates than more remote populations (126, 128).

WHAT ARE THE RISKS OF ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT?

The environmental resistome is vast, ancient, and mobilizable. As such it presents a risk to the development of resistance in pathogens and subsequent drug failure. There is ample evidence to show that the resistance elements currently circulating in pathogens belong to gene families that have their origins in the environment. Given the immense numbers of environmental bacteria and associated resistance genes accumulated over geologic time, it is a reasonable assumption that environmental bacterial genomes are the source reservoir of antibiotic resistance. That said, there are few concrete examples where the direct link between genes found in the clinic and those found in the environment is unimpeachable. The emergence of the *qnr* fluoroquinolone resistance genes, now widely circulating on mobile elements in pathogens, likely originated in environmental *Shewanella* species (120). Similarly, the ESBL of the CTX-M family are thought to have emerged from bacteria of the genus *Kluyvera* (125). Examination of the genomic context of the majority of resistance elements in environmental microbes reveals that these are embedded in genomes and rarely associated with mobilization elements (transposases, integrases, inverted repeat sequences). Yet the history of antibiotics is that resistance, often via mobile genetic elements, inevitably follows use. The environment is clearly a reservoir for these genes, but acquisition by pathogens is not facile. Indeed, were it so, it is unlikely that antibiotics would have emerged as therapeutic successes in the first place. One is therefore left with the hypothesis that use of antibiotics in bulk provides a strong selection for the stochastic capture of resistance genes by mobile genetic elements that can eventually be acquired by pathogens. This gene capture is unpredictable and can occur relatively quickly after antibiotic development, such as in the case of the serine β-lactamases, or only after decades of use, such as with vancomycin resistance. The frequency of these capture events correlates with the gene diversity and burden in the environment. For example, most surveys of resistance genes and phenotypes in nonpathogenic environmental bacteria identify β-lactamases and aminoglycoside-modifying enzymes as highly prevalent, and these were the first resistance elements to be found on mobile elements in previously sensitive pathogenic bacteria (34). It is not unreasonable to hypothesize that antibiotics that show less abundant resistance gene diversity and frequency in the environment will similarly be slower to show resistance in the clinic. This criterion should be a valuable screen in antibiotic discovery to identify which scaffolds to focus on in drug development.

The recognition that the environment is a nearly boundless reservoir of antibiotic resistance has resulted in several studies that seek to estimate the risk of gene transfer to pathogens (6, 14, 93, 97). This examination identifies so-called hot spots of gene transfer (manure, wastewater treatment plants), where mixing of pathogens, mobile elements, and resistance genes is more likely and

therefore has greater potential to affect health (53). The absence of contact between pathogens and environmental bacteria and the lack of enabling mobilization genes and sequences, for example, are mitigating issues that decrease the risk of gene transfer into pathogens. Establishing more sound policies to avoid unnecessary risk of gene transfer, in particular for new antibiotics, should be a priority. Nevertheless, the history of antibiotics has demonstrated time and again that even low-frequency events will occur and antibiotic resistance is inevitable.

CONCLUSIONS

Antibiotics are either products of living organisms or creations of humans in the lab. Regardless of their origins, they are all subject to natural selection. The introduction of thousands of tons of antibiotics into the environment over the last 75 years is unprecedented in the entire history of the planet. Microbes have responded accordingly. Resistance elements, long encoded in their genomes, accumulated and evolved over geologic eras, and in response to microbial exposure over these periods to organic and inorganic toxins, they have been and continue to be mobilized through existing mechanisms as countermeasures to human use of antibiotics. Indeed antibiotics may be profoundly altering how microbes evolve (56). Once in the gene pool of human and animal pathogens, they are easily exchanged and disseminated horizontally through bacterial populations (67). Bacteria have acquired the ability to respond to toxins such as antibiotics over millions to billions of years. The impact on our use of these compounds to improve health is therefore hardly surprising.

Antibiotic resistance is a One Health problem, i.e., one that intricately links humans, animals, and the environment (62, 124). The environments that microbes inhabit—soil, water, air, animal microbiomes—are a vast reservoir of genes capable of responding to all conceivable antibiotics. Mobilization of these genes into pathogens is only a matter of time, and there are no irresistible antibiotics. What is poorly understood is what makes some resistance elements more successful than others in pathogens. The environmental resistome is much more genetically and mechanistically diverse than what has emerged in pathogens. By understanding the evolution and origins of resistance, their fundamental molecular mechanisms, how their associated genes mobilize, and what properties the successful ones share in pathogens, we can begin to address the challenge of drug discovery in the resistance era.

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