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Antibiotic resistance in the environment: a link to the clinic?

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The emergence of resistance to all classes of antibiotics in previously susceptible bacterial pathogens is a major challenge to infectious disease medicine. The origin of the genes associated with resistance has long been a mystery. There is a growing body of evidence that is demonstrating that environmental microbes are highly drug resistant. The genes that make up this environmental resistome have the potential to be transferred to pathogens and indeed there is some evidence that at least some clinically relevant resistance genes have originated in environmental microbes. Understanding the extent of the environmental resistome and its mobilization into pathogenic bacteria is essential for the management and discovery of antibiotics.

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Introduction: antibiotic resistance is a global phenomenon

Unlike other classes of drugs, antibiotics are distinctive in that their use precipitates their obsolescence by selecting for resistant microbes. This reality compounds the challenges inherent in the discovery of new antibiotic drugs, which include for example the difficulty in identifying suitable bioactive chemical matter that can traverse microbial membranes [1]. These challenges have conspired to make new antibiotic discovery a low priority for the pharmaceutical industry despite a growing clinical need [2].

Resistance is inevitable and understanding the origins, evolution, and dissemination of antibiotic resistance elements provides vital information for antibiotic drug discoverers. For example, by taking some of the unpredictability out of the emergence and mechanisms of resistance and by helping to guide compound screening, inform lead identification, and aiding in selection of priority candidates for clinical trials.

One of the most important discoveries made by Davies in 1973 [3], which was elaborated in the following years by several other investigators [4,5], is that antibiotic resistance is not restricted to pathogenic bacteria. Rather environmental microbes that are either non-pathogenic such as antibiotic producing bacteria or opportunistic pathogens such as *Pseudomonas aeruginosa* are often very drug resistant in comparison to the bacteria typically associated with disease. The role of these organisms as potential reservoirs of resistance genes is only now becoming a focus of research [6•,7,8].

There are $\sim 5 \times 10^{30}$ bacteria on the planet [9]. The vast majority of these microbes are not pathogenic. These organisms interact with a multitude of chemicals in the environment produced by other bacteria, fungi, plants, animals, as well as those derived from abiotic processes. Calculation of a very rough estimate of the biologically derived chemical diversity in the soil is instructive. One gram of soil holds 10^7–10^9 bacteria comprised of 4000–10,000 species [10]. Schloss and Handelsmann [10] have shown that in an Alaskan soil sample, that 5.8% of the bacteria are actinobacteria. These are well known to be producers of bioactive compounds, therefore 1 g of soil can contain ~580 different species of actinobacteria. Genome sequencing of members of this group of bacteria that originate in the soil such as the *Streptomyces*, reveal numerous (20–30) gene clusters per genome that can produce different molecules. One gram of soil therefore can include 11,600 (580 × 20) genetic programs encoding bioactive small molecules. This estimate only considers the ~6% of bacteria that are actinobacteria, the remaining 94% also can produce small molecules to a greater or lesser extent, therefore this estimate of chemical diversity is at the very low end of what can be produced by soil bacteria. Furthermore, it does not consider bioactive chemicals produced by other organisms in the soil (fungi, protists, plants, invertebrates). Environmental microbes therefore are embedded in an environment rich in chemical diversity.

Exposure to environmental chemicals is not a contemporary phenomenon. Fossilized stromatolite mats have been identified that are >3.45 B years old [11]. These are the remnants of cyanobacterial communities, which are prodigious producers of bioactive chemicals. Terrestrial plants are also exceptional producers of chemicals and these emerged over 0.4 B years ago [12]. Bioactive chemical diversity in the environment is therefore ancient and

vast. By virtue of their impact on cellular process, such compounds are drivers of natural selection and, as a result, a contributing factor in the evolution of microbial genetic diversity. In the face of this exposure to environmental chemicals over millennia, it is not surprising that environmental microbes have evolved complex machinery for sensing, responding to, and metabolizing small molecules.

Genome sequencing has revealed that all bacteria, even *Mycoplasma genitalium* with a genome size of only 0.58 Mb, have genes dedicated to respond to small molecules of external origin for both nutrition and protection [13]. These genes encode receptors, transport and efflux proteins, immunity elements, and enzymes evolved to chemically modify specific compound classes. All of these classes of genes can be co-opted by microbes in response to cytotoxic molecules including antibiotics. These chromosomal genes are the wellspring of resistance and are pervasive in bacteria from the environment to the clinic.

The environmental antibiotic resistome

The focus of the majority of research in antibiotic resistance over the past six to seven decades has been on its association with pathogenic bacteria. Given what we now know about the dispersal of resistance genes in non-pathogenic bacteria, this focus on pathogens actually neglects the majority of genes associated with resistance. The concept of the antibiotic resistome has been advanced to serve as a framework for understanding the ecology of resistance on a global scale [14]. The resistome consists of all antibiotic resistance genes including those circulating in pathogenic bacteria, antibiotic producers, and benign non-pathogenic organisms found either free living in the environment or as commensals of other organisms.

The first systematic study of the extent of the resistome was published in 2006 and consisted of a survey of spore forming microbes isolated from various soils [15••]. In this work, 480 strains of bacteria were screened vs. 21 different antibiotics representing a broad range of mode of action and chemical classes of drugs. These included natural products and their semi-synthetic derivatives, as well as completely synthetic compounds. Furthermore, the antibiotics spanned molecules that had been introduced in the clinic at the beginning of the antibiotic era over 60 years ago to those that had only very recently been clinically approved. Regardless of mode of action or origin, resistance was observed to all antibiotics in these strains of soil bacteria. Furthermore, all strains were multi-drug resistant. Molecular mechanisms of resistance included known strategies used by pathogenic bacteria as well as novel ones. This study offered an informative sampling of the resistance burden in the environment outside the clinic and demonstrated that antibiotic resistance is widespread in environmental bacteria.

Dantas and colleagues expanded on this work and isolated a number of soil bacteria capable of subsisting on antibiotics as sole carbon sources [16••]. Eighteen antibiotics were sampled including natural product and synthetic antibiotics and microbes that could breakdown these for subsistence were identified in 11 soil samples. Furthermore, many of these organisms were found to have intrinsic multi-drug resistance phenotypes. This highly important discovery demonstrates that there is a previously unanticipated chemical modification and degradation capacity in environmental microbes. This could be a reservoir for new resistance mechanisms but also a source of enzymes with the capacity to modify chemically complex compounds such as antibiotics. Such enzymes could find use as catalysts for new compound synthesis.

In another study designed to investigate whether antibiotic resistance genes in transgenic plants were at risk of increasing the resistance gene burden of soil microbes, Demanèche and colleagues surveyed cultivatable β-lactamase resistant organisms from soils growing transgenic Bt176 corn (containing the β-lactamase gene *bla*-TEM116) and control soils growing non-transgenic corn, or from a non-agricultural (prairie) environment [17]. Their results showed conclusively that β-lactamase producing bacteria were prevalent in soil regardless of whether the soil was agriculturally used or whether transgenic plants containing the *bla*TEM116 gene were grown there for 10 consecutive years. In fact, the native prairie soils had a higher burden of β-lactam resistant bacteria in this study.

A limitation of the culture-based surveys of environmental bacteria is that they only sample a small fraction (perhaps as little as 1%) of the total microbial diversity. In the Demanèche study, bacterial culture was supplemented by PCR-amplification and sequencing of *bla*-TEM genes (Table 1) from total soil DNA, which identified a number of alleles and pseudogenes relevant to understanding the distribution of resistance in the soil [17]. This approach requires prior knowledge of the sequences of genes of interest and does not readily allow for the discovery of novel genes. Functional metagenomics is an orthogonal approach that overcomes these limitations [18]. In this strategy pioneered by the Handelsman group, total DNA from soil samples is isolated and expression libraries are constructed followed by screening on various antibiotics. Using this approach, several novel antibiotic resistance genes have been identified and characterized including aminoglycoside acetyltransferases [19•,20], β-lactamases [20,21], fenicol efflux proteins [22], and inactivators of the anticancer antibiotic bleomycin [23]. This phenotypic screening methodology is a powerful approach in the discovery of novel genes. The strategy does suffer from the disadvantage that gene expression is necessary for selection during screening.

Table 1

Glossary of antibiotic resistance elements

Gene	Associated enzyme activity	Targeted antibiotics	Notes
bla	Hydrolase	β-Lactams	Widely distributed with many varieties, e.g. TEM, SHV
ctx-M	Hydrolase	β-Lactams	Linked to resistance to cephalosporins containing an oxyimino group such as cephotaxime
cat	Acetyltransferase	Chloramphenicol	
sul	Dihydropterate synthase	Sulfonamides	Drug resistance version of susceptible cellular enzyme
tetO, *tetW*	Immunity protein	Tetracyclines	Bind to the bacterial ribosome and decrease affinity for tetracyclines
qnrA	Immunity protein	Fluoroquinolones	Protects DNA gyrase from fluroquinolone antibiotics
vanR, *vans*, *vanH*, *vanA*, *vanH*	Cell wall biosynthesis proteins	Glycopeptides	Synthesis of an altered peptidoglycan with reduced affinity for glycopeptide antibiotics

In order to overcome this limitation, pyrosequencing coupled with hybridization of probes of known resistance genes has been used [24].

In a rare quantitative study of changes in antibiotic resistance from soil over time, Graham and colleagues isolated DNA from agricultural soils collected from the Netherlands from 1940 to 2008 and used qPCR to quantify the presence of selected antibiotic resistance genes [25]. The authors found that levels of all resistance genes investigated rose over time from the pre-antibiotic era (1940) to the present. The rise from the 1970s to the present was especially dramatic for tetracycline and β-lactam resistance elements, likely reflecting changes in agricultural practice and demonstrating the enrichment of resistant organisms with the modern use of antibiotics. Furthermore, the authors identified genes encoding the CTX-M extended spectrum β-lactamase in soils before this gene emerged as a major clinical problem [26]. This supports the suggestion that these genes were resident in environmental organisms such as *Kluyvera* before emerging in the clinic [27••].

Aquatic environments have also been sampled for resistance elements and this literature has been recently reviewed [28,29]. Like soil, resistant organisms in water can represent the intrinsic resistance of normal aquatic microbial populations. Alternatively, they can be the result of contamination by anthropogenic sources such as runoff from agricultural areas or from the use of antibiotics in aquaculture. Contamination of water from the use of antibiotics in agriculture and aquaculture is a significant problem that is increasing the burden of resistance genes and pathogens such as *Escherichia coli* and *Salmonella* in the environment. Discharge of drug resistant pathogens into the environment certainly has an impact on the overall movement of resistance through microbial communities and into the clinic, however it is the result of human activity rather than an intrinsic component of environmental bacteria. In a fascinating example of the latter, a study of water samples from water treatment plants and of tap water in cities in Michigan and Ohio using both culture-dependent and culture-independent methods revealed a significant proportion of bacteria resistant to amoxicillin, ciprofloxacin, chloramphenicol, tetracycline, gentamicin, rifampin and sulfisoxazole [30]. Furthermore, resistance genes *bla*TEM, *bla*SHV, *cat*, *cmr*, *sulI*, *sulII*, *tetO* and *tetW* were detected in most samples by qPCR. The resistance in culturable bacteria is likely associated with normal environmental aquatic microbes rather than pathogens, which are killed during water treatment and therefore the risk (if any) to the general public is minimal. However this study does reveal some interesting findings that deserve additional investigation in terms of water treatment practices that may contribute to resistance. For example, in an independent study, *bla*TEM-harboring microbes in sewage were seen to be enriched following treatment [31].

Resistance is not limited to the surface of the globe. In studies of sub-surface bacteria isolated between 173 m and 259 m in depth from two sites, Brown and Balkwill have identified a number of antibiotic resistant isolates, the majority of which were multi-drug resistant [32]. Given the location of collection, it is unlikely that these bacteria have been subjected to human sources of antibiotics. It will be of great interest to know if the molecular mechanisms of resistance displayed by these bacteria are known or novel given their estimated 3 M years of separation from terrestrial microbes.

Antibiotic resistance in animals

The environment not only consists of water and soil but also includes plants, animals and their associated microbes. Several studies have shown that antibiotic resistant bacteria can be isolated from a variety of animals including mammals such as wild boars [33,34] and rodents [35], birds [36–38], fish [39], and insects [40,41]. These bacteria have either been acquired by scavenging on or by exposure to human-associated material (and one study of mammalian species indicates that increased exposure to humans increases prevalence of antibiotic resistant microbes [42]), or they are part of the normal commensal flora of these animals.

Allen and colleagues used functional metagenomics of gypsy moth gut microbial DNA to identify several antibiotic resistance genes [41], which suggests that these genes are intrinsic to the microbiota of this insect. This is consistent with a study by the Church group that investigated both culturable bacteria and gut metagenomic libraries from two human volunteers that showed that commensal microbes harbored a large number of antibiotic resistance elements [43].

Additionally, agricultural practices are having a major impact on the distribution of resistance elements in the environment. Numerous reports have linked antibiotic use or husbandry practices and isolation of resistant bacteria in the production of swine [44,45], poultry [46,47] and beef [48,49]. In some cases, a direct correlation between resistance in farm animals and infection or colonization in humans has been reported, for example, Ref. [44]. The diminished use of antibiotics in 'organic' farming, which would predict a drop off in infectious resistant pathogens, has yet to be conclusively linked to a decrease in resistance prevalence [50]. Furthermore, fruits and vegetables can also be the source of pathogens, many of which are antibiotic resistant, that originate in food handling, irrigation, and manuring practices [51–53]. These sources of resistance elements all contribute to the global resistome.

Conclusions: the clinical impact of environmental resistance

The evidence is now clear that the environment is a vast reservoir of resistant organisms and their associated genes. This resistome is part of the fabric of the global microbial population and predates human use of antibiotics. The question then arises, does the environmental resistome intersect with the resistome of pathogens or are they distinct? Most resistance genes found in pathogens are acquired through horizontal gene transfer (HGT) via mobile genetic elements such as plasmids. Plasmids have been found in bacterial collections of pathogens that predate the antibiotic era and these do not contain resistance genes [54•]. However, these plasmids rapidly became vectors of resistance genes following the wide spread use of antibiotics. In the absence of direct evidence, the source of these resistance genes has been speculative. However, there is growing evidence that antibiotic resistance genes in pathogenic organisms have arisen in the environmental resistome. For example, as noted above, the CTX-M extended spectrum β-lactamase appears to have come from chromosomal genes of environmental genus *Kluyvera* [27••]. The *qnrA* gene associated with plasmid-linked fluorquinolone resistance that has emerged globally has an environmental reservoir in the aquatic bacterium *Shewanella algae* [55]. The gene cluster that confers resistance to the glycopeptide antibiotic vancomycin in enterococci and staphylococci consists of two regulatory genes, *vanR* and *vanS*, and three enzyme-encoding genes, *vanH*, *vanA*, and *vanX*, and have been identified in environmental bacilli [56], in glycopeptide producing bacteria [5], and in non-producing environmental strains [57]. These examples clearly link resistance in the environment with the clinic.

What is absent in the field are quantitative studies that measure the rate of HGT between organisms and identification of the environmental factors that contribute HGT that leads to emergence of resistance in pathogens. No doubt exposure to antibiotics either via agricultural practice, wastewater treatment, etc., are contributing factors, but there may be many others as well. The next challenges in this area of research will be to establish the conditions that contribute to HGT of resistance genes from the environment to the clinic. Another question is why certain resistance genes are more successful in pathogens than others? Current evidence shows that there are frequently multiple mechanisms of resistance in environmental bacteria for different classes of antibiotics, yet often only a select few emerge in the clinic. This knowledge will be vital in predicting the emergence of resistance and its spread providing clinicians, farmers, regulators, and drug discovers vital information for the management of current and future antibiotics.

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