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Environmental and clinical antibiotic resistomes, same only different

Gerard D Wright

The history of antibiotic use in the clinic is one of initial efficacy followed inevitably by the emergence of resistance. Often this resistance is the result of the capture and mobilization of genes that have their origins in environmental reservoirs. Both antibiotic production and resistance are ancient and widely distributed among microbes in the environment. This deep reservoir of resistance offers the opportunity for gene flow into susceptible disease-causing bacteria. Not all resistance genes are equally successfully mobilized, and some dominate in the clinic. The differences and similarities in resistance mechanisms and associated genes among environments reveal a complex interplay between gene capture and mobilization that requires study of gene diversity and gene product function to fully understand the breadth and depth of resistance and the risk to human health.

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The history of antibiotics shows unequivocally that they are all susceptible to resistance. This reality makes antibiotics unique in comparison to drugs for other therapeutic areas. Failure to recognize this fact has resulted in the inappropriate use of antibiotics, the subsequent emergence of untreatable multidrug resistant pathogens, and now a growing gap between the supply of new drugs and the demand in the clinic [1]. Going forward, critical questions for the long-term use of new antibiotics in therapy include: When will resistance emerge? By what mechanism(s)? In which organisms? Are there means to anticipate and mitigate it?

Resistance occurs through many mechanisms including (1) prevention of antibiotic penetration into the cell, (2) active efflux from the cell, (3) modification, protection, or replacement of the molecular target, and (4) enzymatic inactivation or modification of the drug [2]. Each of these mechanisms can arise spontaneously through mutation in bacterial populations. Antibiotics that are particularly prone to resistance via mutation, with frequencies $>10^{-8}$, are generally eliminated in the preclinical discovery and development stage since it is probable that resistance will emerge during treatment of an infection. Alternatively, resistance genes can be imported into a previously susceptible bacterial cell via horizontal transfer (HGT). HGT occurs through the uptake of environmental DNA by competent organisms, through conjugation with other bacteria, or via phage infection. The relative frequencies of these mechanisms in bacterial populations, especially in non-clinical environments, are not well known. Resistance genes that are on mobile elements such as plasmids or transposons that can move laterally by conjugation are particularly concerning in the clinic since these can propagate even between bacteria of different genera. In contrast, there is no consensus that transduction via phage or that uptake of environmental DNA play essential roles in the rapid dissemination of antibiotic resistance in clinical settings, though they likely have some role in the spread of resistance.

Defining resistance

Resistance to antibiotics involves the marshaling of specific genetic elements that enable bacteria to survive a concentration of antibiotic that otherwise would result in inhibition of growth. Aspects of cell physiology such as the presence of the relatively impermeant outer membrane of Gram-negative bacteria or growth modes for example the establishment of biofilms are not generally regarded as bona fide resistance mechanisms, even though they often provide protection from high levels of antibiotics. In fact, this physiology may have evolved for protection against environmental insults including toxic molecules such as antibiotics [3,4]. Resistance is context specific and therefore annotating resistance genes can be challenging and contributes to confusion in resistance gene databases [5•]. For example, all bacteria have some contingent of efflux proteins, and many confer resistance, but only when overexpressed. In the absence of gene expression data, is it meaningful to annotate these as resistance elements? Bacteria may degrade antibiotics as carbon and nitrogen sources and are thus 'resistant' [6–8], but it this meaningful

resistance? Such nuances challenge the definitions of antibiotic resistant organisms.

From the perspective of understanding the origins of resistance and the links between resistomes in various environments, a functional definition of resistance is appropriate. Here I define resistance genes as those encoding proteins or RNA that can be biochemically determined to confer resistance in an otherwise sensitive organism. This definition, therefore, includes genes that may be transcriptionally silent in an organism but which, if captured and mobilized, will confer resistance. Such a definition enables the exploration of the resistance potential of genes and therefore includes the evolutionary and ecological capacity of genes and gene families to be directed under natural selection to confer resistance. It is essential to realize that this functional definition, which must be based on rigorous biochemical analysis, does not provide any guidance regarding the risk of mobilization of such genes into clinically significant organisms [5•]. As a result, it is relevant to use functional strategies to investigate the resistance potential of resistomes, with the understanding that the mechanisms and odds of whichever specific genes, if any, that may migrate to the clinic or organisms of particular concern are currently opaque.

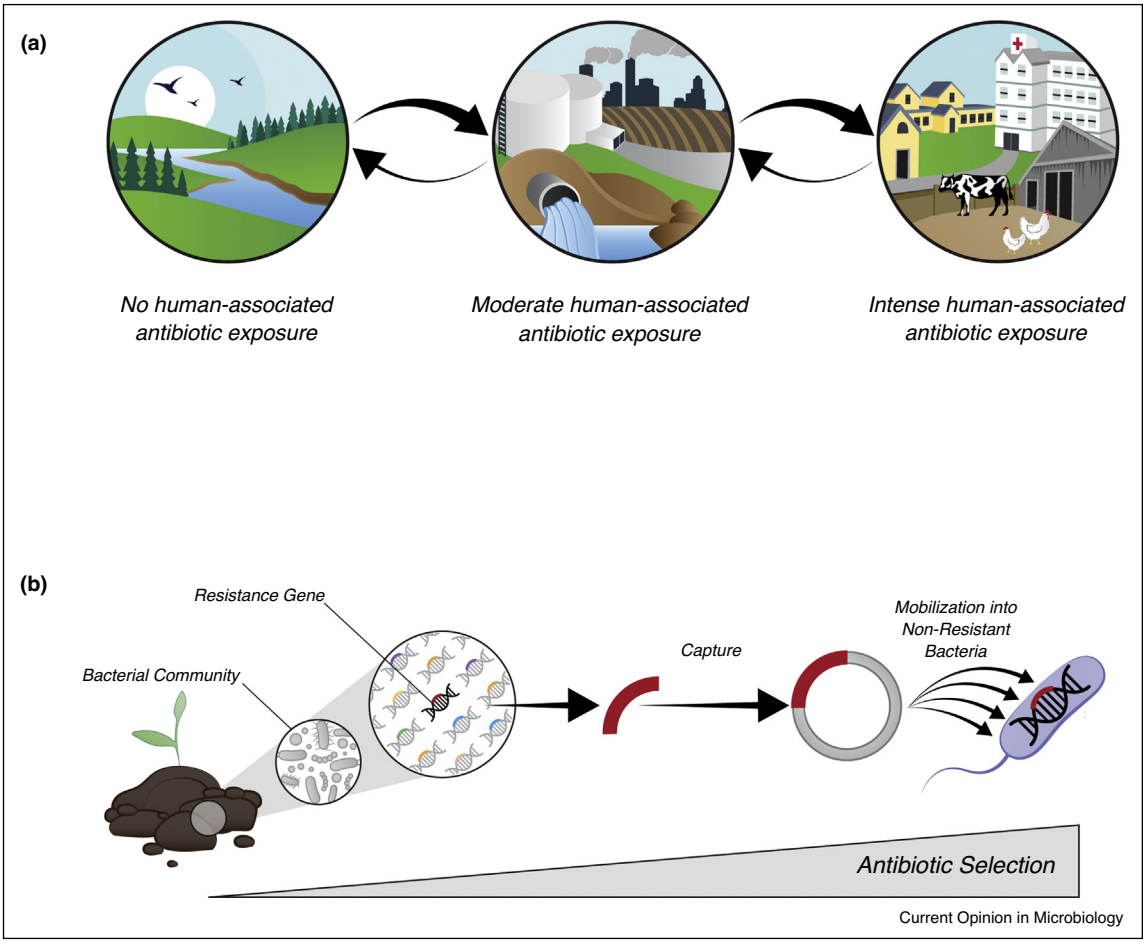
There is now sufficient circumstantial and direct evidence to confirm that the majority of resistance genes that are acquired by pathogens through HGT have their origins in bacteria that reside principally in environments not significantly impacted by human use of antibiotics [9,10,11•,12,13,14•,15–17]. I use the term 'pathogen' for bacteria that are typically associated with disease and 'environment' for organisms that are generally not linked to infections. It is understood that this definition is arbitrary and dependent on many factors [18], for example, the immune status of the host or the genetic makeup of the microorganism, nevertheless the terms pathogen and environment are useful qualifiers when thinking about resistance gene flow and risk to patients. From the perspective of resistance, one can differentiate the 'environment' into three interconnected spheres of different microbial communities (Figure 1a). First are pristine environments namely untreated soils, water, samples that predate the antibiotic era, and so on, where there are few pathogens, but where a vast genetic diversity of non-infectious microbes are dominant, many of which produce small molecules with antibiotic activity and thus harbor resistance genes. Second are human-impacted external environments where antibiotic residues and resistance elements are linked to human use of antibiotics, for example, wastewater treatment plants, manured soils, landfills, and so on. Here microbial communities can be a mix of bacteria associated with disease and benign environmental organisms. Third are environments where intensive human use of antibiotics occurs, hospitals and other patient care centers, farms, and so on, and where we see a higher proportion of human and animal pathogens. The evidence suggests that many resistance elements that are common in pathogens originate in pristine environments and move through bacterial populations through selection resulting from antibiotic use [9,10,11•,12,13,14•,15–17].

Genes can migrate between environmental resistomes through initial episodic, and likely highly rare, capture of resistance genes followed by mobilization by agents of HGT (Figure 1b). Resistance genes that are already on mobile plasmids are of more concern for the mobilization into bacterial populations associated with disease. A recent survey of 922 soil organisms from the Earth Microbiome Project revealed that over 40% had at least one plasmid, some of which carried resistance genes [19•]. Over 68% of these plasmids were predicted to be mobile, offering a straightforward mechanism for gene dissemination between resistomes. That said, resistance genes were much more common on bacterial chromosomes, and therefore presumably of less risk of mobilization.

Diversity and similarity of resistomes

Several surveys of resistance from a variety of environments demonstrate that mechanistic diversity and frequency of resistance elements in bacteria sourced from pristine environments exceeds what is generally found in disease-causing organisms. In fact, pre-antibiotic era strains of common bacterial pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Neisseria gonorrhea*, *Salmonella enterica*, and so on, are intrinsically highly sensitive to antibiotics. In contrast, environmental bacteria, even those that are opportunistic pathogens such as *Pseudomonas* and *Acinetobacter* species, are comparatively inherently well protected against antibiotics, even in the absence of exposure to human-associated use of antibiotics. Put another way, the organisms that, at least in the pre-antibiotic era, were more closely associated with human diseases (many of which are components, even if transient, of human and animal microbiomes) are in general much more intrinsically sensitive to antibiotics than bacteria that typically reside in non-human/animal ecosystems. For example, an analysis of the Murray collection [20] of strains of Enterobacteriaceae collected from human infection sites dating between 1917–1954, revealed that while conjugative plasmids were common, antibiotic resistance was rare [21]. On the other hand, an analysis of ancient metagenomic DNA from Yukon permafrost (permanently frozen sediments) dated to 30 000 years ago, identified genes conferring resistance to β-lactam, tetracycline, and glycopeptide antibiotics [22] and a survey of 480 contemporary soil actinomycetes revealed that all were multidrug resistant [23]. The evidence is consistent that even before the antibiotic era, resistance elements were abundant in pristine environments but rare in pathogenic bacteria.

Figure 1



Environments and resistance gene flow.
(a) A simplified view of various environments linked to microbial communities and antibiotics. Pristine environments (left) have little exposure to human use of antibiotics, and bacterial communities are dominated by organisms that are not generally associated with diseases in health animals and humans. Wastewater treatment plants, agricultural soils, and industrial lands are intermediate environments (middle) with some exposure to human-derived antibiotics and often mix potential pathogens and benign environmental bacteria. Hospitals and farms that raise food animals are environments where there is intense use of antibiotics and significant numbers of human and animal pathogens. Gene exchange can occur between all these environments. **(b)** Mobile elements can stochastically capture resistance genes, which can move among various organisms and ecosystems. Increased antibiotic use offers the selective pressure for genes to flow toward once sensitive microorganisms.

The link between antibiotic production by environmental bacteria and resistance was first reported by Benveniste and Davies in 1973 [9]. The logic of this discovery is now obvious in that bacteria that produce antibiotics must also have the means to prevent self-intoxication [24]. Over the years, it became increasingly evident that these resistance mechanisms in producing organisms are often identical to those found in pathogens [25]. The depth of resistance in environmental microbes is apparent in surveys of phenotypic and genotypic resistance, for example, Refs. [7,12,23,26–29]. Some of these genes are identical to those circulating in pathogens [11•], but most are not. Why is there a difference in genetic diversity and what mechanisms underpin which resistance genes will be successfully migrated to pathogens? Understanding these differences is vital to guide drug discovery efforts and to the surveillance of resistance elements in the environment and clinic.

The known knowns, known unknowns, and unknown unknowns of resistance

Identifying known resistance genes (known knowns) and new members of resistance gene families (known unknowns) is straightforward from genome and metagenome sequences. Algorithms such as the RGI of the CARD database [30] and Resfams [12] mine DNA sequence information and can quickly identify such resistance elements. However, the identification of new genes and mechanisms (unknown unknowns) requires additional biochemical experimentation. The use of

functional genomics, where resistance genes are identified from libraries of expressed proteins, is especially useful in this regard given the powerful selection that antibiotic resistance provides in screening of genomic libraries [31–34].

For example, to comprehensively describe the intrinsic resistome of a multidrug-resistant environmental organism, we studied *Paenibacillus* sp. LC231 [35••]. This strain was collected from the Lechuguilla Cave in New Mexico, an underground ecosystem isolated from the surface for ~4 M years [26]. Genome sequencing of *Paenibacillus* sp. LC231 did not reveal any resistance genes identical to those in databanks (known knowns) but did identify several new members of well-studied aminoglycoside, macrolide, chloramphenicol, streptogramin, linezolid, and rifamycin resistance gene families (known unknowns), which were then biochemically verified. While these elements accounted for some of the antibiotic resistance phenotypes observed, resistance to several other antibiotics could not be accounted for in the bioinformatic analysis of the genome. Using functional genomics, we identified new resistance genes (unknown unknowns) associated with resistance to the peptide antibiotic bacitracin, in addition to pleuromutilins, lincosamides, aminoglycosides, kasugamycin, tetracycline, capreomycin, and mupirocin [35••]. This study of a single organism offers a glimpse of the depth and breadth of the resistome of pristine environments and speaks to the long evolutionary history of the interaction of antibiotics and microorganisms. No doubt, similar studies that reconcile resistance phenotype with genotype using complementary genomic and biochemical tools will continue to reveal a complex and mechanistically diverse global resistome.

Two examples of mechanistic diversity across resistomes: β-lactams and rifamycins

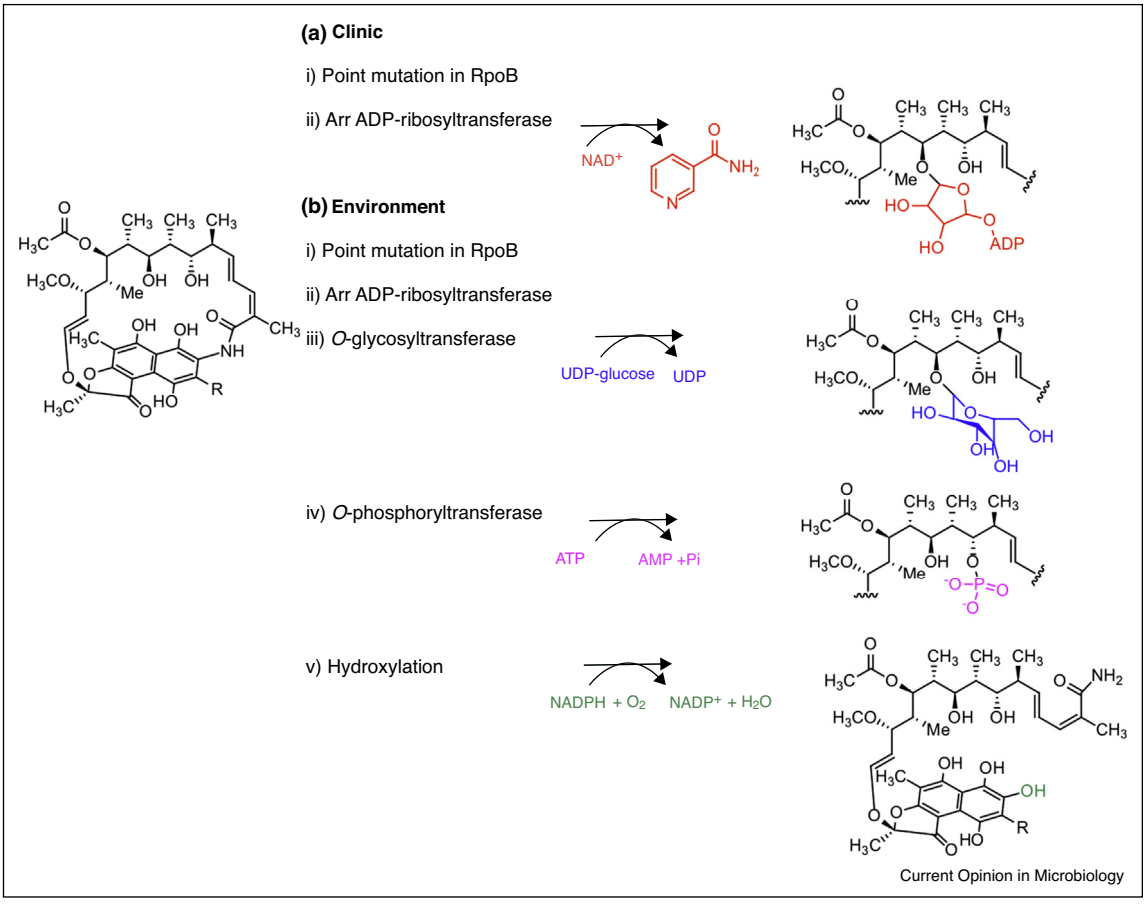
The β-lactam antibiotics – penicillins, cephalosporins, carbapenems, and monobactams, – remain the most widely used antibiotics in the world today [10]. The principal mechanism of resistance to these drugs is through the expression of β-lactamases that hydrolytically cleave the β-lactam ring by one of two general biochemical mechanisms: Ser-mediated formation of a labile acyl-enzyme intermediate (Ambler class A, C, D), or Zn^{2+}-dependent activation of an active site water molecule (Ambler class B) [36]. The first report of β-lactam resistance mediated by β-lactamases in 1940 [37] predates the clinical use of these antibiotics, which explained the original observation by Fleming, twelve years earlier, that some bacteria were intrinsically resistant to the activity of penicillin [38]. We now understand that many bacteria harbor chromosomally encoded β-lactamases such as those of the AmpC family in Enterobacteriaceae [39] and OXA in Acinetobacter [40].

Direct evidence of β-lactamases that predate clinical use include the presence of TEM-related genes in 30 000 year-old permafrost [22] and in 10 000 year-old marine sediments [41], a PC1 orthologue from a *Staphylococcus saprophyticus* isolated from a human skeleton excavated from a grave from Byzantine Troy [42], and a 700 year-old metallo-β-lactamase from a *Brucella melitensis* genome isolated from a 14th Century human bone fragment [29,43]. The first description of the presence of a mobile TEM β-lactamase in was in 1965 [44] followed quickly by its biochemical characterization [45]. Since then, this highly successful family of resistance elements has spread across the globe and become dominant; there are 213 orthologues entered in the CARD database (retrieved May 1, 2019; card.mcmaster.ca). The reason(s) why this gene family, in particular, is so successful is unknown but may have to do with its frequent association with transposable elements and the fact that it was among the first to emerge in clinical isolates.

In the environment, the genetic diversity of β-lactamases eclipses that which is found in the clinic, where it already accounts for the largest number of known resistance genes. One of the first functional metagenomic efforts to study β-lactamases in pristine environments identified several new members of all Ambler class β-lactamases including a unique bifunctional enzyme with C-terminal class C and N-terminal class D homologies [46••]. This unusual enzyme brings β-lactamases into a small group of bifunctional antibiotic resistance enzymes [47]. A similar metagenomic study identified new members of class A and class C β-lactamases [34]. A recent comparative metagenomic study of 232 shotgun metagenomes from 10 different environments (glaciers, ocean, farms, human feces) emphasized the broad diversity of β-lactamases and that families of genes that frequently appear in clinical settings (TEM, CTX-M, OXA, GES) are ubiquitously found in many non-clinical environments, thereby acting as a vast potential reservoir for gene distribution [48].

Rifamycin antibiotics such as rifampin offer another example of the broad diversity of resistance across various environments. In medicine, the semi-synthetic rifamycin antibiotic rifampin is primarily used for the treatment of tuberculosis. The causative agent, *Mycobacterium tuberculosis*, is increasingly resistant to rifampin due to mutation in the target RNA polymerase (*rpoB*). Another mechanism that circulates in some Gram-negative bacteria on mobile elements and is chromosomally encoded in some non-tuberculosis mycobacteria is NAD-dependent antibiotic ADP-ribosylation encoded by *arr* genes (Figure 2) [49]. In the environment, genetic and mechanistic diversity is much more significant. For example, in a survey of 480 soil actinomycetes, 49 were resistant to rifampin at 20 μg/mL [23]. Many of these had canonical rifamycin resistance mutations in *rpoB*, but 40% were able to inactivate the antibiotic by either glycosylation, phosphorylation, or

Figure 2



The mechanistic diversity of rifamycin antibiotic resistance.
(a) Clinical resistance is dominated by point mutation in the target RpoB. The ADP-ribosyltransferase Arr enzymes also circulate in these microbial communities. **(b)** In pristine environments, additional mechanisms include *O*-glycosylation, *O*-phosphorylation, and hydroxylation leading to ring opening.

decomposition [50]. Glycosylation is mediated by a unique member of the UDP-glucose glycosyltransferase family [50]. Functional studies identified a previously unknown phosphorylation resistance mechanism catalyzed by a rare member of the ATP-dependent dikinase family, RPH, that transfers the β-phosphate to substrates, unlike typical kinases that transfer the γ-phosphate (Figure 2) [51,52]. Both glycosylation and phosphorylation occur on essential hydroxyl groups of the antibiotic that interact with RpoB, thus accounting for resistance. Finally, the decomposition mechanism was determined to be initiated by ROX, a FAD-dependent monooxygenase that catalyzes a remarkable C–N bond cleavage resulting in the linearization of the cyclic rifampin, destroying the 3-dimensional shape necessary to engage RpoB (Figure 2).

Other examples of remarkable genetic diversity of resistance elements in pristine environments include the large family of Qnr genes conferring resistance to quinolone antibiotics [53,54], aminoglycoside modifying enzymes [33,35••], macrolide kinases [14•,55], and alternative peptidoglycan synthesis conferring resistance to glycopeptide antibiotics [56].

Conclusions

Antibiotics are ancient and so is resistance. Consequently, there exist a wide variety of resistance mechanisms in pristine and pre-antibiotic environments. Capture of genes in this deep reservoir, driven by the powerful selection of human antibiotic use, has facilitated and accelerated the natural gene flow among microorganisms. The result is that once drug-susceptible pathogens are now resistant and often multidrug resistant. What is increasingly clear is that pristine environments offer a large number of genes and mechanisms to draw from. What is unclear, is why antibiotics such as rifampin, which has multiple resistance mechanisms in pristine environments, have few in the clinic (outside *M. tuberculosis*,

which does not undero HGT). Similarly, why are gene such as TEM so successful?

It is essential to remember as well that resistance can evolve in real time in the clinic. The emergence of enzyme-catalyzed ciprofloxacin resistance through mutation and selection of an aminoglycoside acetyltransferase, AAC(6')-Ib-cr, is a striking example [57]. What is needed are in-depth studies on the genetic and mechanistic diversity of resistance outside of clinical settings. This will enable increased understanding of the depth and breadth of resistance and particularly for new antibiotics that will come to the clinic, offer an early warning system for the inevitable emergence of resistance.

Conflict of interest statement

Nothing declared.

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