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Call of the wild: antibiotic resistance genes in natural environments

Heather K. Allen[*‡], *Justin Donato*[*], *Helena Huimi Wang*[§], *Karen A. Cloud-Hansen*[*], *Julian Davies*[§] *and Jo Handelsman*[||]

Abstract | Antibiotic-resistant pathogens are profoundly important to human health, but the environmental reservoirs of resistance determinants are poorly understood. The origins of antibiotic resistance in the environment is relevant to human health because of the increasing importance of zoonotic diseases as well as the need for predicting emerging resistant pathogens. This Review explores the presence and spread of antibiotic resistance in non-agricultural, non-clinical environments and demonstrates the need for more intensive investigation on this subject.

Antibiotic resistance genes in human pathogens such as methicillin-resistant *Staphylococcus aureus*[1] have become notorious because they confound the tools that are used to treat disease (FIG. 1). In particular, resistance determinants in pathogens are commonly encountered after the introduction of an antibiotic to clinical use, and treating human pathogens with antibiotics directly affects the frequency of resistance to those antibiotics in these pathogens[1–4].

The presence of antibiotic resistance elements in pathogenic bacteria is made all the more problematic because of the prevalence of horizontal gene transfer, the process by which bacteria acquire genes from the environment[5]. Many of the known antibiotic resistance genes are found on transposons, integrons or plasmids, which can be mobilized and transferred to other bacteria of the same or different species. There is evidence of the transfer of resistance elements to known human commensal bacteria and pathogens[6,7], and gene transfer in the human intestinal microbiome is extensive[8].

What are the sources and reservoirs of these transferable genes? A full understanding of the pressures and circumstances that lead to the evolution and dissemination of antibiotic resistance genes in pathogens is impossible without a detailed examination of the origin and role of resistance genes in natural environments. This Review discusses the environmental sources of antibiotic resistance, the functions and roles of resistance genes in microbial ecology and the ways by which those genes may be disseminated in response to human antibiotic use.

Selection pressures in the environment

Antibiotics are essential for the treatment of bacterial infections in humans and animals; it is therefore a top priority to preserve their efficacy. For decades, clinicians and scientists have called for the prudent use of antibiotics, in an effort to slow the development and epidemic spread of resistance[9–11]. Prudent use of antibiotics in humans demands that physicians establish that a bacterial infection is responsible for the patient's symptoms before an antibiotic prescription is written. By contrast, in agriculture antibiotics are used in the absence of acute infection. Some of the same antibiotics that are used to treat human pathogens, such as amoxicillin and erythromycin, are also used to treat disease, promote growth and improve feed efficiency in animals[12] (BOX 1). Just as in hospital settings, the agricultural use of antibiotics selects for antibiotic resistance, arguably in a more widely disseminated fashion owing to the farm-wide administration of prophylactic antibiotics in feed and water. Antibiotics from both urban and agricultural sources persist in soil and aquatic environments, and the selective pressure imposed by these compounds may affect the treatment of human diseases[13,14]. As another example, the prophylactic use of antibiotics in fish farms has led to a rise in the number of resistant bacteria[15]. Strikingly, these resistant bacteria can transfer the resistance genes to human pathogens[16]. The selection pressure applied by the antibiotics that are used in clinical and agricultural settings has promoted the evolution and spread of genes that confer resistance, regardless of their origins (FIG. 2).

[*]*Departments of Bacteriology and Plant Pathology, University of Wisconsin–Madison.*
[‡]*Microbiology Doctoral Training Program, University of Wisconsin–Madison, 1550 Linden Dr., Madison, Wisconsin 53706, USA.*
[§]*Department of Microbiology and Immunology, University of British Columbia, 2350 Health Sciences Mall, Vancouver, British Columbia V6T1Z3, Canada.*
[||]*Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut 06520, USA.*
Correspondence to J.H.
e-mail: *jo.handelsman@yale.edu*
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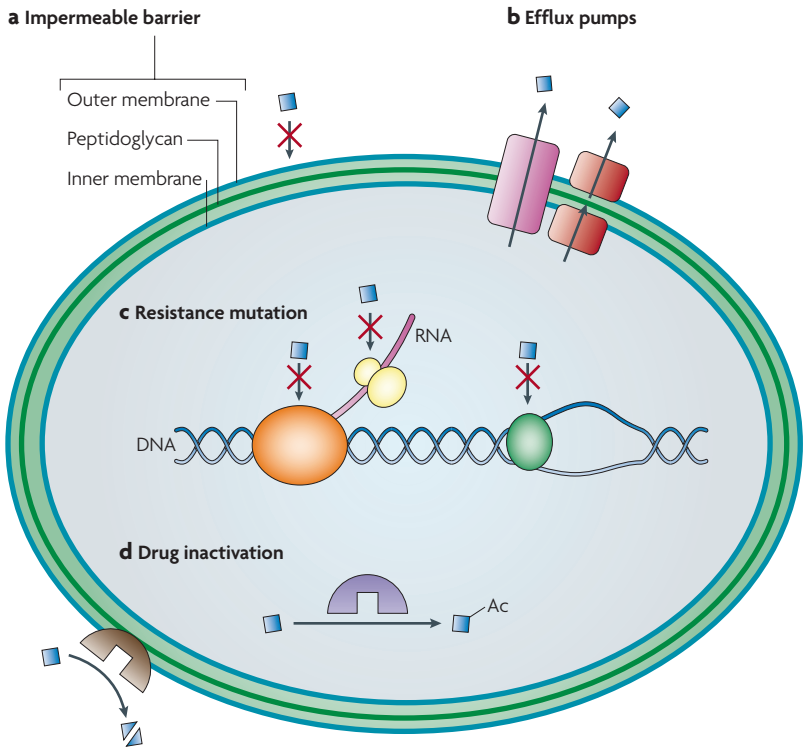

Figure 1 | **Mechanisms of antibiotic resistance in a Gram-negative bacterium.** **a** | Impermeable barriers. Some bacteria are intrinsically resistant to certain antibiotics (blue squares) simply because they have an impermeable membrane or lack the target of the antibiotic. **b** | Multidrug resistance efflux pumps. These pumps secrete antibiotics from the cell. Some transporters, such as those of the resistance–nodulation–cell division family (pink), can pump antibiotics directly outside the cell, whereas others, such as those of the major facilitator superfamily (red), secrete them into the periplasm. **c** | Resistance mutations. These mutations modify the target protein, for example by disabling the antibiotic-binding site but leaving the cellular functionality of the protein intact. Specific examples include mutations in the gyrase (green), which cause resistance to floroquinolones, in RNA polymerase subunit B (orange), which cause resistance to rifampicin, and in the 30S ribosomal subunit protein S12 (encoded by *rpsL*) (yellow), which cause resistance to streptomycin. **d** | Inactivation of the antibiotic. Inactivation can occur by covalent modification of the antibiotic, such as that catalysed by acetyltransferases (purple) acting on aminoglycoside antibiotics, or by degradation of the antibiotic, such as that catalysed by β-lactamases (brown) acting on β-lactam antibiotics. Ac, acetyl group.

The use (and misuse) of antibiotics by humans is probably not the only selective pressure for antibiotic resistance in natural microbial communities: compounds and conditions that occur in these communities may provide additional selection pressures. Indeed, most antibiotics are produced by strains of fungi and bacteria that occur naturally in all environments, including soil[17] (FIG. 2). Most antibiotic-producing strains carry genes encoding resistance to the antibiotics that they produce[18,19], and these genes are usually found in the same gene cluster as the antibiotic biosynthesis pathway genes[17,20]. Antibiotics produced in the environment may exert selective pressure on neighbouring organisms. However, it is difficult to determine the natural concentrations of antibiotics in soil microcosms or the extent of the selective pressure that they may pose.

Accidental resistance genes. The presence of various potentially offensive compounds and conditions in nature might select for specific or nonspecific mechanisms of antibiotic resistance (FIGS 1,2). Bacteria cultured from the marine air–water interface were shown to be more highly resistant to antibiotics than bacteria cultured from the bulk water[21], and numerous conditions, including radiation and pollution, that may select for antibiotic resistance in this habitat have been suggested, although the mechanisms of cross resistance to antibiotics are unknown. Some genes confer antibiotic resistance but are likely to have other primary roles in the environment[22]. Certain classes of efflux pumps, for example, offer general mechanisms of resistance, because they pump various toxins, such as heavy metals and other toxic molecules, out of cells[23,24]. For some chromosomally encoded multidrug resistance pumps, such as those of the resistance–nodulation–division family, antibiotic resistance is now thought to be an associated function of the primary role that they serve in the environment[24], which might be, for example, to provide tolerance to toxic compounds. The microbial communities of insect guts that had no known exposure to antibiotics contain efflux pumps that confer resistance to antibiotics when transferred to *Escherichia coli*[25,26]. Both oil fly larvae and gypsy moth larvae ingest compounds that might stress microorganisms; oil fly larvae eat organic solvents, and gypsy moth larvae feed on diverse plants that produce various toxic compounds. In *Shewanella oneidensis*, a bacterium that lives in sediment, the multidrug efflux transporter gene *mexF* enhances fitness in the environment and confers resistance to chloramphenicol and tetracycline in the laboratory[27]. Further information on the roles of efflux pumps in bacteria can be found in REF. 28.

The movement of antibiotic resistance genes

Physical forces. Physical forces, such as those created by wind and watershed, are important drivers of the spread of antibiotic resistance genes (FIG. 2). Antibiotics and their resistance genes have been widely distributed in the environment since before the introduction of antibiotic chemotherapies, but human activities have probably increased the prevalence of resistant bacteria in the air and water. As a result, antibiotic resistance is more common in *E. coli* and *S. aureus* isolates from air inside the home than in isolates from outside[29,30], although a study of sulphonamide resistance in *E. coli* detected more antibiotic-resistant isolates in dust outside homes than in dust inside homes in Mexico[31]. Marine and freshwater ecosystems also contain bacteria from many sources, including antibiotic-resistant bacteria from anthropogenic sources[32]. Even bacteria from environments that are thought to be stationary, such as soil, can be moved by the forces of nature; one example is the intercontinental transport of bacteria on desert dust[33].

Animals. Wild animals provide a biological mechanism for the spread of antibiotic resistance genes (FIG. 2). Proximity to human activities influences the antibiotic resistance profiles of the gut bacteria of wild

Resistotype
The antibiotic resistance genotype and phenotype of a bacterium.

mammals, which live in densely populated microbial habitats in which antibiotics select for resistance. Ninety percent of the bacterial isolates from mice and voles captured in rural England were resistant to β-lactam antibiotics[34]. By contrast, the faecal enterobacteria of wild elk, deer and voles in Finland have almost no resistance[35]. For the most part, Finland is less densely populated than England; therefore, these findings might suggest that human activities influence antibiotic resistance in bacterial communities in wild animals, although other influences that affect the frequency of antibiotic resistance cannot be eliminated (including differences in the testing methodologies used or variation in the intrinsic antibiotic resistance of the isolate populations). Likewise, a study of *E. coli* isolates from wild animals in Mexico and in Australia found a higher frequency of antibiotic resistance in the isolates from Mexico[36]. It was suggested that this difference may be due to widespread human settlement and use of antibiotics in Mexico along with possible selection pressures from the host animals[36]. Other studies demonstrate a similar association — African baboons and apes that are in contact with humans harbour more antibiotic-resistant enteric bacteria than those that dwell in areas that are remote from human activity[37,38].

Wild birds carry a reservoir of antibiotic-resistant bacteria with the potential for long-distance dissemination. Birds, and migratory waterfowl in particular, can travel great distances and inhabit a wide variety of environments, from agricultural lagoons to remote mountain lakes, and can potentially spread resistance genes along the way. Proximity to human activity increases the number of the antibiotic-resistant bacteria that are associated with wild birds. Gulls and geese nesting near waste or agricultural water harbour more antibiotic-resistant *E. coli* than do birds associated with unpolluted water[39,40]. Antibiotics also seem to affect resistance in remote bird populations: in arctic birds, 8% of *E. coli* isolates were recently found to be resistant to at least 1 of 17 antibiotics tested, and 4 were resistant to 4 or more antibiotics[41]. One isolate was resistant to cefadroxil, cefuroxime and cefpodoxime, a pattern that is common in clinical isolates[41]. Many birds breed in the arctic and migrate to up to six continents. They probably acquire antibiotic-resistant bacteria either from environments that are under human influence or from other birds that contact those environments, illustrating the great geographical distances that can be travelled by resistance genes that are associated with human selective pressures.

One study looking at the effect of the proximity of human activity on the presence of antibiotic resistance genes found no antibiotic-resistant *E. coli* from remote animal populations but notable populations of antibiotic-resistant *E. coli* in animals that were proximal to anthropogenic activity[42]. Animals that live with humans, including pets such as cats and dogs, are reservoirs of antibiotic-resistant bacteria as a result of both antibiotic treatment for disease and the transfer of resistant bacteria from humans[43]. The data suggest that exposure to antibiotics has affected antibiotic resistance in the enteric bacteria of wild animals, although many more studies using standardized methods are required to define this impact. More complete profiles of antibiotic resistance in wild animals will contribute to our understanding of the origins and roles of antibiotic resistance genes in natural intestinal microbial communities, which, in turn, will help us manage emerging zoonotic diseases.

Humans. Antibiotic-resistant bacteria have been found in even the most secluded communities, although proximity to dense human populations affects the antibiotic resistotypes that are found. For example, although antibiotic-resistant *Salmonella* and *Shigella* species were isolated from humans living far from Kathmandu, Nepal, there were far fewer than were isolated from humans living near to the city[44]. High levels of antibiotic resistance were also found in *E. coli* from an isolated human population in Bolivia[45], and the resistance genes in the remote community (such as β-lactamase TEM (*blaTEM*)-like genes and the aminoglycosyl adenyltransferase gene *aadA1*) closely matched genes from antibiotic-exposed environments[46]. As this population has little access to modern health care, and contact with people outside the community is minimal, the results show that antibiotic resistance in this remote community is entirely due to a diverse array of resistance genes that had immigrated from elsewhere. Despite the barriers, antibiotic resistance genes have been transmitted to the most isolated human populations, where they exist even in the absence of an obvious selection pressure.

Box 1 | **Antibiotics in food production**

Antibiotics are used in diverse settings for food production. Animals are treated with antibiotics for both curing disease and promoting growth[7], fruit trees are often treated prophylactically with antibiotics to control bacterial infections[98], and aquaculture relies on antibiotics to manage infectious disease[15]. In each of these situations, the effects of the antibiotics extend beyond the site of use. Antibiotics applied in animal farming operations leach into waterways and groundwater; in many aquaculture settings, antibiotics diffuse into the water surrounding the pens; and antibiotics sprayed on plants can drift aerially.

The Food and Agriculture Organization (FAO) of the United Nations has the task of monitoring and compiling global statistics about the international regulation of pesticide use. The philosophy and legality of the administration of antibiotics in agricultural settings vary among countries. In 1985, Sweden enacted the Feeding Stuffs Act, which outlawed the administration of antibiotics to livestock for growth promotion[99], in stark contrast to the many industrial countries that use vast quantities of antibiotics for this purpose. Even countries that have adopted similar overall patterns of regulation vary in the specifics. For example, the United Kingdom, the United States, Norway, Mexico, India and Indonesia have approved the use of oxytetracycline in aquaculture, whereas among these same countries only Mexico and Indonesia permit the use of enrofloxacin[100].

Some governments have taken action in response to public concern about the use of antibiotics in agriculture. Consumers are also directly shaping farm practices, leading McDonald's to purchase only antibiotic-free beef for its globally sourced restaurants[101]. As more large corporations and governments follow suit, antibiotic application practices in agriculture might be reduced and standardized globally.

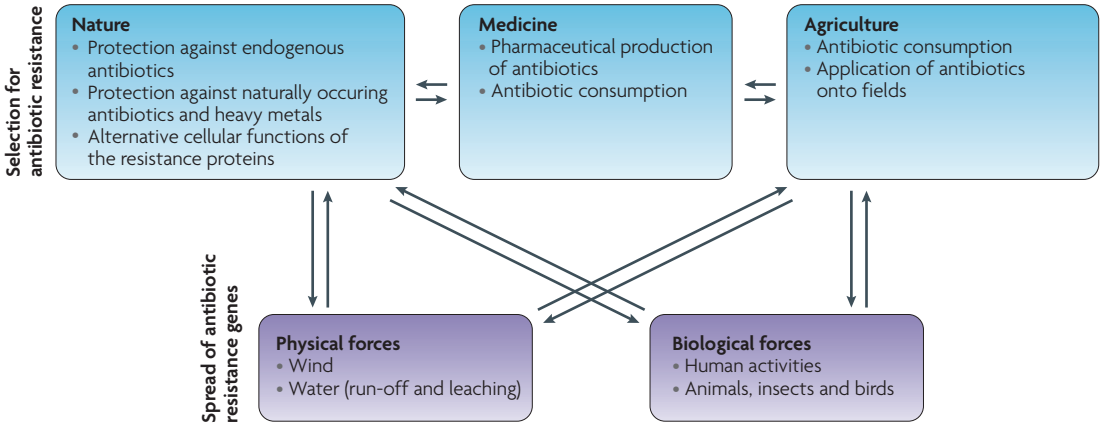

Figure 2 | **Sources and movement of antibiotic resistance genes in the environment.** Resistance genes exist naturally in the environment owing to a range of selective pressures in nature. Humans have applied additional selective pressure for antibiotic resistance genes because of the large quantities of antibiotics that we produce, consume and apply in medicine and agriculture. Physical and biological forces also cause widespread dissemination of resistance genes throughout many environments.

Antibiotic resistance in natural communities

Little is known about the selection pressures on antibiotic resistance genes in the era before antibiotics were turned into pharmaceuticals or in remote environments with little direct human contact. A more comprehensive understanding of the natural roles of putative antibiotic resistance genes will provide important information on their origins and functions.

The pre-antibiotic era. The only environments that are truly exempt from the influence of human antibiotic use existed before the antibiotic era. The time before the introduction of sulphonamides, which occurred in the late 1930s, can be considered 'antibiotic naive', in the sense that no industrial production of antibiotics took place. However, heavy metals were used for disease treatment for centuries prior to the use of antibiotics, and this may have selected for genes encoding both heavy-metal and antibiotic resistance[47]. Retrospective studies show that resistance genes were present in bacteria that did not produce antibiotics before the widespread dissemination of the drugs. Out of 30 *E. coli* strains that were lyophilized before 1950, 4 were resistant (to various degrees) to the 8 tested antibiotics, and each resistance element could conjugate into *E. coli*[48]. In another analysis of 433 enterobacterial strains collected from around the world between 1917 and 1952 (known as the Murray collection), 24% could transfer plasmids and 11 strains were resistant to ampicillin or tetracycline, although the resistance was not conjugative[49]. More recent analysis of antibiotic resistance profiles from enteric bacteria collected before and after the introduction of antibiotics mirrors these findings[50]. Thus, determinants of antibiotic resistance existed naturally and were probably subject to horizontal transfer long before the extreme selection pressure that was imposed in the antibiotic era. This predisposition for the genetic exchange of resistance elements is certain to have facilitated the rapid outgrowth of antibiotic resistance in pathogenic bacteria.

Soil. Owing to the movement of antibiotics and resistance genes on the wind and on feathers, it is unlikely that any environment can be considered truly pristine. However, despite the movement of soil particles by physical forces, soil itself is a stationary complex, and some soils are far removed from human influences. Thus, studies of antibiotic resistance in soil show that environmental bacteria harbour antibiotic resistance genes independently of human activities.

Culturable bacteria in soil harbour genes encoding enzymes that degrade or otherwise inactivate antibiotics. Bacteria that grow on antibiotics as the sole carbon and nitrogen sources include: *Pseudomonas fluorescens* grown on streptomycin[51]; *P. fluorescens*[52], *Burkholderia cepacia*[53] and eight unidentified strains[54] grown on penicillin; an unidentified *Streptomyces* sp. isolate and *Streptomyces venezuelae* grown on chloramphenicol[55,56]; a *Flavobacterium* sp. isolate grown on chloramphenicol[57]; and various bacteria, mostly of the phylum Proteobacteria, grown on various antibiotics[58]. In addition to antibiotic degradation, culturable soil bacteria use many mechanisms of resistance to antibiotics. In the most comprehensive study to date on antibiotic resistance in one soil species, over 400 actinomycetes cultured from forest, agricultural and urban soils were found to have highly varied resistance profiles; moreover, some exhibited resistotypes that had not been seen before, such as inactivation of telithromycin by a novel structural modification[59].

The roles of antibiotics and resistance in nature

The discovery of the great therapeutic potential of microbial compounds in both the laboratory and the clinic led to the preconception that antibiotic activity must be important and widespread in nature. This led to an almost complete disregard of the other potential functions of natural products from microorganisms. On the basis of limited genomic studies, it is thought that microbial populations are capable of

Pristine
Unspoiled or unpolluted by human activities.

producing a wide range of bioactive small molecules (the parvome[60]), only a handful of which have been isolated, identified and used as antibiotics and other types of therapeutics. Bacteria of the phylum Actinobacteria[61], a huge taxonomic group that is characterized by a high genomic GC content and that comprises diverse genera, all produce complex bioactive small molecules. It can be estimated that actinobacteria make millions of such molecules. Most of these molecules cannot be detected under laboratory conditions, and the few that have been identified include the most important antibiotics. It has been suggested that antibiotics have been produced for over 500 million years, dating back to the Cambrian period and the emergence of vertebrate fish[62]. Antibiotic-like molecules, or at least their component parts, are likely to be even older than this — the non-protein amino acids that are found as components of peptide antibiotics have been detected in meteorites and other primordial sources[63].

The roles of antibiotics in microbial communities. Using war as a metaphor for the interaction of microorganisms, the activities of antibiotics in the laboratory and in clinical applications led to the assumption that these molecules have hostile roles in nature. The number of such bioactive molecules in any given environment must provide a considerable armamentarium. However, the *in situ* concentrations of the compounds with antibiotic activity have never been measured, and there are few ecological examples of probable antibiotic functions for microbial products in nature. One example is the fungus-growing ant system, in which ants carry an antibiotic-producing actinomycete (a *Pseudonocardia* sp.) on their cuticle and use this bacterium specifically for biocontrol of the fungal garden parasite, *Escovopsis* sp.[64,65]. A second example is in the biocontrol of the causative agent of potato scab, *Streptomyces scabies* str. RB4, by the antibiotic-producing suppressive strain, *Streptomyces diastatochromogenes* str. PonSSII[66]. Many factors may contribute to disease suppression, but the fact that antibiotic-resistant strains of the pathogen are restored in their ability to cause disease is evidence for a direct role for antibiosis[66]. Because of the sparse evidence for widespread antibiotic-mediated killing in nature, it is important to investigate the role of sublethal doses of antibiotics in microbial communities.

The demonstration of quorum-sensing reactions in a range of microbial species opened up a profitable field of investigation that has had implications for research into various aspects of bacterial lifestyles, such as pathogenesis, community structure and biofilm formation[67]. These all involve the production of specific bioactive compounds that, at low concentrations, activate biochemical pathways in one or more target organisms. Interestingly, some autoinducers used in quorum sensing have antibiotic activity at higher concentrations and may also provoke changes in eukaryotic host organisms or tissues[68]. These studies have led to the realization that bioactive small molecules (other than amino acids, sugars and nucleic-acid bases) have important roles in microbial biology[69]. For example, quinolones, phenazines and pheromones are bioactive small molecules that exist widely in nature, and they each possess a range of biological functions.

In recent years, several antibiotics and other bioactive molecules (such as bacteriocins) have been tested for activity at concentrations below those needed for the inhibition of cell growth. Almost all of the tested compounds exhibit hormesis[70,71]. This suggests that the compounds constitute a new form of signalling network, in which the receptors for the small molecules are cytoplasmic macromolecular structures such as ribosomes and the DNA replication, RNA replication and cell wall synthesis complexes[72]. Note that many of these receptors were previously identified as the inhibitory targets for the bioactive molecules. The binding of the ligands to their receptors at low concentrations initiates a profusion of different transcription patterns, depending on the nature of the ligand and the target. Modulation of host transcription leads to metabolic and behavioural changes in the microorganisms, as has been described in several reports[70,73]. These changes can be assumed to be signalling responses that adjust metabolism in mixed microbial communities.

The role of antibiotic resistance genes in microbial communities. As mentioned above, the production of an antibiotic is associated with the presence of genes encoding one or more self-protection processes; antibiotic biosynthesis gene clusters always encode one or more potential resistance proteins that are either specific to the compound being made (for example, they modify the compound or target) or multifunctional (for example, efflux systems). In addition to the so-called self-resistance function, the resistance genes that are contiguous with the biosynthesis genes could be involved in regulation of the biosynthesis pathway. One study that supports this concept found genes encoding export proteins embedded in the actinorhodin biosynthesis pathway of *Streptomyces coelicolor*[19], but is this yet another example of anthropocentric reasoning? Evidence shows that antibiotic resistance genes are common in natural environments and existed, even on plasmids, before the use of antibiotics. Phylogenetic analyses date the origin of serine β-lactamases at over 2 billion years ago and suggest that many of these enzymes have been plasmid encoded for millions of years[74].

Another form of resistance in isolates that do not produce antibiotics is mutation of the target gene product, which reduces or prevents inhibition by antibiotic binding. However, antibiotic resistance may not be the only consequence of these mutations, as they are often pleiotropic. These types of mutations have been found frequently in environmental bacteria, which are therefore, presumably, unresponsive to a specific small molecule signal in the environment. When soil bacteria were screened for isolates that were resistant to known antimicrobials such as the fluoroquinolones, it was found that the isolates had independent alleles of the DNA gyrase subunit A gene (*gyrA*) with different

Parvome
The range of biologically active, low-molecular-mass (< 5 kDa) compounds that are produced by defined biosynthetic pathways in bacteria, yeast, plants and other organisms.

Antibiosis
An interaction between microorganisms involving a small molecule that is produced by one organism and detrimental to the other.

Hormesis
A dose-dependent response phenomenon shown by bioactive compounds and drugs, such that they have contrasting activities at low (subinhibitory) and high (inhibitory) concentrations.

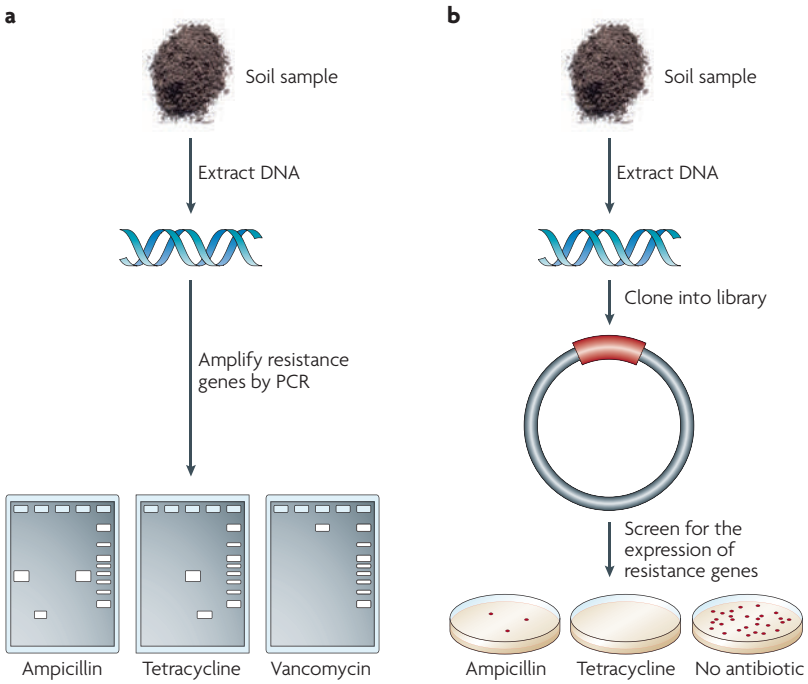

Figure 3 | **Detecting antibiotic genes in natural samples. a** | A PCR-based approach. DNA is extracted from bacteria in a soil sample and resistance genes are amplified with resistance gene-specific primers and detected by gel electrophoresis. **b** | Functional metagenomics. DNA is extracted from soil samples and cloned into libraries. These libraries are introduced into *Escherichia coli* or another amenable host, and the transformants are tested for drug resistance.

levels of resistance[75]. Subsequent studies have shown that spontaneous mutations causing resistance often lead to a range of metabolic phenotypes, including variations in the ability to use different carbon, nitrogen or phosphate sources for growth[76]. In addition, mutations in ribosomal proteins that conferred resistance to streptomycin, spectinomycin or macrolides were found to cause a range of altered phenotypes, as shown by phenotypic array studies (H.H.Wang and J. Davies, unpublished observations). Bacteria carrying these mutations were selected under laboratory conditions in the presence of an antibiotic, but in the environment the selection could alternatively be the ability to grow on an available peptide or the presence of a particular carbon or phosphorus source. Thus, the widespread presence of antibiotic-resistant strains in the environment may have arisen in response to various selective pressures. It is clear that more extensive studies of the roles of natural resistant strains and their diverse phenotypes are needed.

In addition to the alternative cellular and environmental roles of known antibiotic resistance genes, other types of genes that contribute to resistance have been recognized. Studies undertaken at the species level revealed an extensive set of genes that contribute to the antibiotic resistance phenotype but that primarily encode proteins with other functions in the cell (the so-called intrinsic resistome). In *E. coli*, for example, 4,000 random single-gene knockouts were screened for hypersensitivity to antibiotics[77,78]. Of these, 140 knockouts were identified that increased the sensitivity to at least 1 of 7 antibiotics[77]. Some genes were implicated in resistance to many types of antibiotics (for example, an insertion in the gene encoding the DNA helicase exonuclease V subunit-γ (*recC*) increases susceptibility to ciprofloxacin, rifampin, sulphamethoxazole and metronizadole) and others were involved in resistance to a specific antibiotic (for example, an insertion in the transcriptional regulatory gene *phoP* increases susceptibility to ampicillin)[77]. Similar studies were performed in *Pseudomonas aeruginosa*[79,80] and *Acinetobacter baylyi*[81], revealing that there is little overlap between the intrinsic resistomes of different organisms. These results implicate possible targets for antibiotic potentiators and illustrate the breadth of the genes, even in a single organism, that can contribute to the overall environmental antibiotic resistome.

Challenges in studying natural resistance

Detecting antibiotic resistance genes. The application of culture-independent approaches, such as PCR and metagenomics, to the study of antibiotic resistance in the environment has uncovered the vast diversity of antibiotic resistance genes in soil bacteria (FIG. 3). A prairie soil was analysed by PCR for the presence of genes encoding TEM-type β-lactamases. Numerous polymorphisms of the TEM sequence were discovered in this prairie soil as well as in soil containing transgenic plants[82]. However, certain *Taq* polymerases have been shown to be contaminated with DNA that encodes TEM-type β-lactamases[83] (B. Converse and J. Handelsman., unpublished observations), indicating that precautions need to be taken when using PCR methods to detect TEM-related β-lactamases. PCR has also been used to detect vancomycin[84] and gentamicin[85] resistance genes in unamended soils, revealing sequences that are closely related to known genes. Primers for *tetM*, one of the known tetracycline resistance genes, were used to analyse unamended garden soils by PCR but detected no related tetracycline resistance genes[86]. PCR is therefore a powerful tool to detect potential antibiotic resistance genes in soil environments.

In contrast to PCR, functional metagenomics can be used to query an environment for genes that are not closely related to known resistance genes. Functional metagenomics is the study of the collective genome of a group of organisms by cloning DNA directly from an environmental sample into a host organism (often *E. coli*) followed by screening or selecting for the desired function[87]. When applied to a Wisconsin remnant oak savannah soil site, functional metagenomics yielded diverse and new antibiotic resistance genes, including nine aminoglycoside resistance genes and one tetracycline resistance gene[88]. Six of the aminoglycoside resistance genes encoded 6′-*N*-acetyltransferases, and a phylogenetic analysis of these enzymes at the amino acid level showed that they cluster together and are divergent from previously known 6′-*N*-acetyltransferases. The same method was applied to the metagenome of an Alaskan soil that was distant from anthropogenic activities, in

Potentiator
A compound or molecule that augments the activity of an antibiotic.

Box 2 | **Human exposure to antibiotic-resistant bacteria in wildlife**

Potential routes for human contact with wild animals and their microbiota, which may contain antibiotic-resistant strains, include:

- Translocation of wildlife into suburban areas owing to game release, habitat destruction, pollution and changes to water storage, irrigation or the climate.
- Ecotourism, hunting and camping.
- Exotic foods, wet markets, bushmeat and game farms.
- Exotic pets and the long-distance transport of live animals.
- Zoos, aquaria, wildlife safari parks and circuses.
- Trapping or rearing of fur-bearing animals.

search of resistance to β-lactam antibiotics. This study revealed 13 β-lactamases (including 1 bifunctional enzyme) that represent each of the 4 known classes of β-lactamases but that are deeply divergent from and ancestrally related to the β-lactamases found in clinical isolates[89]. Interestingly, none of the functional β-lactamases from the Alaskan soil metagenome were related to TEM-type β-lactamases, which is in contrast to the results of the PCR-based investigation of prairie soil. Functionally characterizing the metagenomes of bacterial communities from a variety of environments will expand our knowledge of the potential sources and novel alleles of antibiotic resistance genes.

Part of the challenge of addressing and reviewing the big questions surrounding the ecology of antibiotic resistance genes in the environment is that inconsistent methods have been used to monitor the resistance properties of environmental bacteria. Most studies of resistance in bacteria from the environment are based on culturing followed by selection. However, the method of resistance determination is not standardized; therefore, the data on antibiotic resistance in the environment come from studies that have used a range of media types, antibiotic concentrations and incubation periods. This makes it difficult to compare results between environments. For example, Antarctic marine waters contain copious antibiotic-resistant bacteria, including many isolates that are resistant to ampicillin[90]. However, resistance was determined by growth on 50 μg ml^{-1} ampicillin, which is not consistent with other studies of antibiotic resistance in aquatic environments or with how resistance is defined for clinical isolates, which are tested for minimum inhibitory concentration according to standard protocols[91]. The absence of guidelines for resistance studies of environmental bacteria makes it difficult to draw conclusions about a single environment or to make comparisons between environments.

The inconsistencies in the methodologies are further aggravated by the infrequent and incomplete monitoring of antibiotic resistance in natural environments. The intestinal microbiota of wild animals, for example, have rarely been examined for carriage of antibiotic resistance genes. Little is known about these bacteria more generally[92], despite the alarming statistic that in the past 2 decades approximately 75% of all types of emerging human diseases came from wildlife[93]. As potential routes for human contact with wild animals expand (for example, through translocation of wildlife into suburban areas owing to habitat destruction and an increase in the exotic food and pet trades), the potential for bacterial transfer also increases (BOX 2). The data on antibiotic resistance in wild animals also show the need for multiple strategies to be used to fill our knowledge gaps. For example, less than 1% of the bacteria in the environment are culturable by standard techniques, although for some environments this estimate is conservative. Despite the known limitations of culturing, nearly all studies of antibiotic resistance in wild animals are based on culturing for enteric bacteria. However, considering that the spread of antibiotic resistance genes by horizontal gene transfer occurs between diverse bacteria with ease and that resistance genes are maintained even in the absence of selection[8,94], it is important to characterize the resistance genes in the entire community, including both culturable and unculturable strains. In addition, culture-independent techniques have the potential to carry microbial ecologists beyond gene definition and into gene expression studies. Techniques such as reverse transcription PCR, when coupled with metagenomics, enrich studies by investigating which genes are expressed across the entire community. Both culture-dependent and culture-independent approaches have their limitations, so combining these approaches will develop the most comprehensive portrait of the resistance profile of a microbial community, which will form the basis for understanding the effects of environmental resistance genes on human pathogens and the role of antibiotic resistance genes in unperturbed communities.

Conclusions

Little is known about the antibiotic resistomes[59] of the vast majority of environmental bacteria, although there have been calls for a greater understanding of the environmental reservoirs of antibiotic resistance and their potential impacts on clinically important bacteria[95,96]. The data on antibiotic resistance before the antibiotic era and in soil highlight how little we know about the ecology of antibiotic resistance genes in the wild. We do not have a complete picture for any environment of all of the types of resistance genes in both the cultured and uncultured community. Soil is particularly challenging to assess, because of its chemical and physical heterogeneity (soil displays variation on a scale of 1 metre or less)[97]. Despite the gaps in our knowledge, it is clear that some organisms and some environments harbour antibiotic resistance genes irrespective of the human use of antibiotics. The prevalence and diversity of resistance genes in the environment inspire hypotheses about the native roles of so-called resistance genes in natural microbial communities. Considering that antibiotic treatment is our primary, and in many cases only, method of treating infectious diseases, we conclude that more detailed studies of environmental reservoirs of resistance are crucial to our future ability to fight infection.

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Competing interests statement
The authors declare no competing financial interests.

DATABASES
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Entrez Genome Project: http://www.ncbi.nlm.nih.gov/genomeprj
Burkholderia cepacia | *Escherichia coli* | *Pseudomonas aeruginosa* | *Pseudomonas fluorescens* | *Shewanella oneidensis* | *Staphylococus aureus* | *Streptomyces coelicolor*

FURTHER INFORMATION
Jo Handelsman's homepage: http://bbs.yale.edu/people/jo_handelsman.profile
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