Review
Future Microbiology

Insights into antibiotic resistance through metagenomic approaches

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The consequences of bacterial infections have been curtailed by the introduction of a wide range of antibiotics. However, infections continue to be a leading cause of mortality, in part due to the evolution and acquisition of antibiotic-resistance genes. Antibiotic misuse and overprescription have created a driving force influencing the selection of resistance. Despite the problem of antibiotic resistance in infectious bacteria, little is known about the diversity, distribution and origins of resistance genes, especially for the unculturable majority of environmental bacteria. Functional and sequence-based metagenomics have been used for the discovery of novel resistance determinants and the improved understanding of antibiotic-resistance mechanisms in clinical and natural environments. This review discusses recent findings and future challenges in the study of antibiotic resistance through metagenomic approaches.

Infectious diseases are the second-leading cause of death globally and the most significant cause of death in children [1,2]. Antibiotics represent one of the largest therapeutic categories used in the treatment of infectious diseases caused by bacteria, but the successful use of any therapeutic agent is compromised by the potential development of tolerance or resistance to that compound from the time it is first employed. In clinical environments, pathogenic and commensal bacteria are challenged with high concentrations of antibiotics and bacteria have become resistant to most of the antibiotics developed [3]. Within the hospital setting, resistance pathogens often emerge within a few years after a new antibiotic is introduced (Figure 1). The spread of resistant bacteria and resistance genes depends on different factors but the major pressure is antibiotic usage [4]. Nosocomial (hospital-linked) infections result in approximately 100,000 deaths and cost more than US$25 billion per year in the USA alone. Worldwide, it is estimated that 5–10% of patients entering hospitals develop an infection as a result of their stay [101]. Despite the problem of antibiotic resistance in infectious bacteria, little is known regarding the diversity, distribution and origins of resistance genes, especially for the unculturable majority of environmental bacteria.

Mechanisms of antibiotic resistance

Resistance to antibiotics can be caused by four general mechanisms (Figure 2) [5]:

- The inactivation or modification of the antibiotic;
- An alteration in the target site of the antibiotic that reduces its binding capacity;
- The modification of metabolic pathways to circumvent the antibiotic effect;
- The reduced intracellular antibiotic accumulation by decreasing permeability and/or increasing active efflux of the antibiotic.

Bacteria can develop resistance to antibiotics by mutating existing genes (vertical evolution) [6,7], or by acquiring new genes from other strains or species (horizontal gene transfer) [8,9]. The sharing of genes between bacteria by horizontal gene transfer occurs by many different mechanisms. Mobile genetic elements, including phages, plasmids and transposons mediate this transfer, and in some circumstances the presence of low levels of the antibiotic in the environment is the key signal that promotes gene transfer [10], perhaps ensuring that the whole microbial community is protected from the antibiotic [11].

Detection of antibiotic resistance

Antibiotic resistance is a highly selectable phenotype and can be detected using growth inhibition assays performed in broth or by agar disc diffusion. In a dilution-based growth inhibition assay, the MIC of an antibiotic can be calculated for each bacterial isolate and the organism is typically interpreted as being susceptible

Keywords
■ aminoglycoside ■ amphenicol ■ antibiotic resistance ■ β-lactam ■ functional metagenomics ■ metagenome ■ sequence-based metagenomics ■ tetracycline

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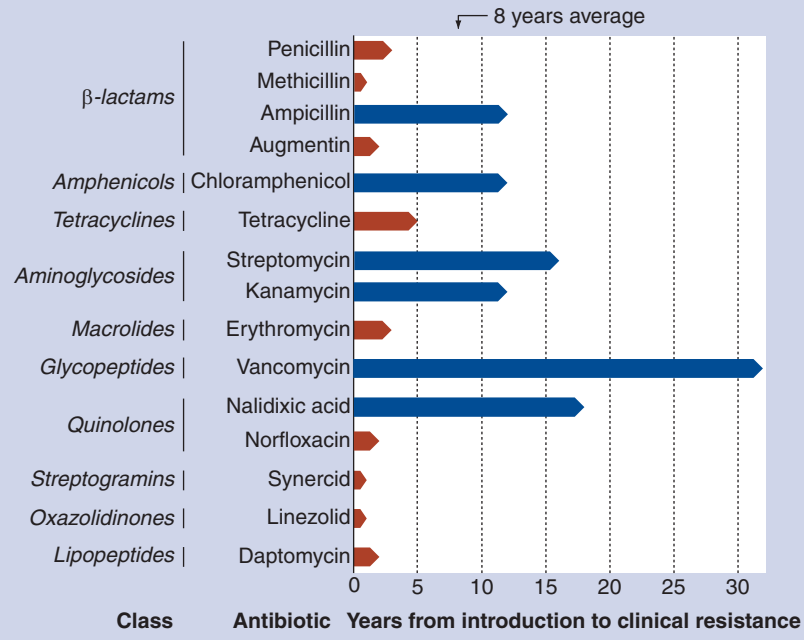

Figure 1. Antibiotic resistance evolution showing the rapid development of resistance for several classes of antibiotics. The bars mark the time from the introduction of an antibiotic to the clinic until the first clinical case of resistance to that antibiotic was reported. The high number of available antibiotics during the 1950s and 1960s attributed to the longer times until the first resistance case was reported (e.g., for nalidixic acid). If the time frames for the same antibiotic differed between the data sources, the shorter time frame was used.
Data taken from [94–97].

or resistant to the antibiotic. Of course, there is a gradation of resistance and some classification schemes include one or more intermediate levels. For clinically important bacteria, diagnostic laboratories perform phenotypic-based analyses using standardized susceptibility testing methods, usually in accordance with those published by the Clinical and Laboratory Standards Institute [102].

To produce results, culture-based approaches can take 1–2 days for fast-growing bacteria, such as *Escherichia coli* or *Salmonella,* but several weeks for slow-growing bacteria, such as *Mycobacterium tuberculosis*. Moreover, culturing only works for a fraction of microbes; although most pathogens can be cultured because of our years of experimental experience, the vast majority of microbes cannot grow outside their host environment, including pathogens such as *Chlamydia* or *Trypanosomes*. Newer molecular detection techniques for resistance such as quantitative PCR (qPCR) [12] or microarrays [13,14] are able to determine the presence of specific resistance genes and have improved diagnosis by providing results within hours. However, these culture-independent approaches only target well-studied pathogens or resistance-causing genes and cannot easily be used for broad-spectrum screening.

Research targeted towards antibiotics for treating infectious diseases and the risks to human health posed by antibiotic resistance have focused mainly on the clinical setting [15,16]. To fully understand the development and dissemination of resistance, we need to address the study of antibiotics and their resistance genes not just in clinics but in natural (nonclinical) environments as well [17–19].

Before next-generation sequencing, antibiotic resistance genes were typically isolated from environmental samples by cloning from cultured bacteria or by PCR amplification [20]. Those methods ignored potential antibiotic resistance reservoirs because most bacteria are not culturable, and PCR detection depends on primers that are based on known resistance genes and does not readily allow for the discovery of novel genes. The development of culture-independent techniques was required to identify novel resistance genes and access the genetic diversity of most bacteria.

Metagenomics is one of the more modern approaches that overcome the limitations of methods based on culturing or amplification (**Box 1**) [21]. This approach is a powerful tool to describe the genetic potential of a community and to identify the types of microbes present in a community, as well as the presence or absence of genes or genetic variations responsible for antibiotic resistance. Using metagenomics, several novel antibiotic resistance genes have been identified, including resistance to β-lactams [22,23], tetracycline [24], aminoglycosides [20,23] and bleomycin [25]. In the next sections, we describe the different metagenomic approaches that are used to identify antibiotic-resistance genes.

Culture-independent study of resistance through metagenomics

In 1985, Pace *et al.* were the first to propose the direct cloning of environmental DNA to classify unculturable microorganisms [26]. The first successful function-driven screening of metagenomic libraries, termed zoolibraries by the authors, was conducted in 1995 [27]. The term 'metagenomics' was coined in 1998 by Handelsman *et al.*, referring to the function-based analysis of mixed environmental DNA species [21]. Initially, metagenomics was used mainly to recover novel biomolecules (especially DNA) from environmental microbial

assemblages. The development of next-generation sequencing techniques led to an alternative approach where a fraction of the DNA in the sample was sequenced *en masse*, without regard to cloning. This approach was sometimes called random community genomics, which also became known as metagenomics. Metagenomic sequencing represented a powerful alternative to rRNA sequencing for analyzing complex microbial communities [20,28] and has a tremendous impact on the study of microbial diversity in environmental and clinical samples. Nowadays, the field of metagenomics can roughly be divided into two different approaches: functional metagenomics and sequence-based metagenomics (Figure 3).

Functional metagenomics

Functional metagenomics involves cloning and heterologous expression of environmental DNA in a surrogate host with coupled activity-based screening to discover functions of genes that might not be obvious from their sequence. By creating a functional metagenomic library in which cloned genomic fragments are expressed and selecting directly for resistance to antibiotics, traditional challenges associated with studying genes of unknown sequence were circumvented. The metagenomic analysis revealed novel antibiotic resistance proteins that were previously of unknown function and unrecognizable by sequence alone. Functional metagenomics has been used to identify genes encoding proteins that inactivate antibiotics, genes encoding multidrug efflux pumps and genes conferring resistance to the folate antagonist trimethoprim. Functional metagenomics has additionally been applied to cultured isolates of a community [29].

Sequence-based metagenomics

Sequence-based metagenomics involves extracting and random sequencing of DNA directly from the environment, including the DNA of uncultured bacteria. Typically, the eukaryotic cells, bacteria, viruses, and free DNA are separated by size (using filtration or centrifugation), and total DNA extracted from the appropriate fraction. A sample of the DNA is sequenced, and that sample is assumed to be a random fraction of the whole community. The metagenomic sequences are then compared to the known sequences that have been accumulated over the years in national and international databanks (reference sequences) to identify resistance genes and/or mutations that are known to cause resistance (Figure 3 & 4). Using a wide range of reference resistance genes, the resistance potential for multiple antibiotics can be predicted from a single metagenome. The metagenomic sequences additionally represent the diversity of the community, including strains that cannot be cultured, valuable information for the study of community changes as a result of antibiotic treatment.

To date, more than one thousand different metagenomes have been sequenced from a large variety of environments, such as soil, ocean and human gut (Figure 5). In addition, extinct species such as the woolly mammoth [30] and the Neanderthals [31] have been analyzed by sequence-based metagenomic approaches.

The volume of sequence data generated in the last few years spawned a new generation of

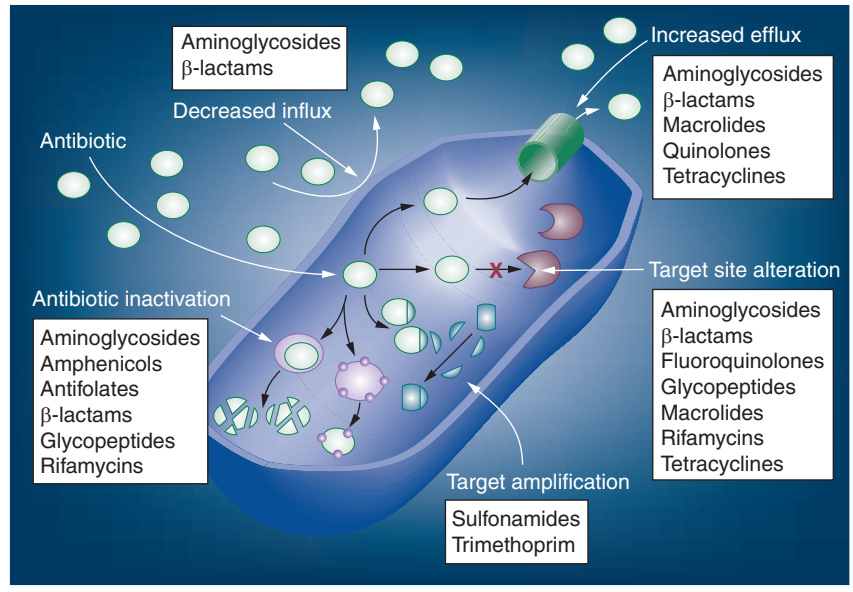

Figure 2. Mechanisms of antibiotic resistance in bacteria. The classes of antibiotics affected by each of the mechanisms are listed in the boxes.

Box 1.

- The generic term 'antibiotic' is used to denote any class of organic molecules that inhibits or kills microbes by specific interactions with bacterial targets, without any consideration of the source of the particular compound or class
- The term 'antibiotic resistome' was proposed for the collection of all antibiotic resistance genes in microorganisms, from both pathogenic and nonpathogenic bacteria
- 'Metagenome' describes the genetic potential of a community
- 'Sequence-based metagenomics' involves extracting and random sequencing of DNA directly from the environment without the need for culturing
- 'Functional metagenomics' involves cloning and heterologous expression of environmental DNA in a surrogate host with coupled activity-based screening
- The term 'microbiome' has been suggested by Nobel laureate Joshua Lederberg to describe the collective genome of our indigenous microbes
- 'Metatranscriptomics' is a culture-independent method by which the collective RNA transcript of an entire community of microorganisms is analyzed

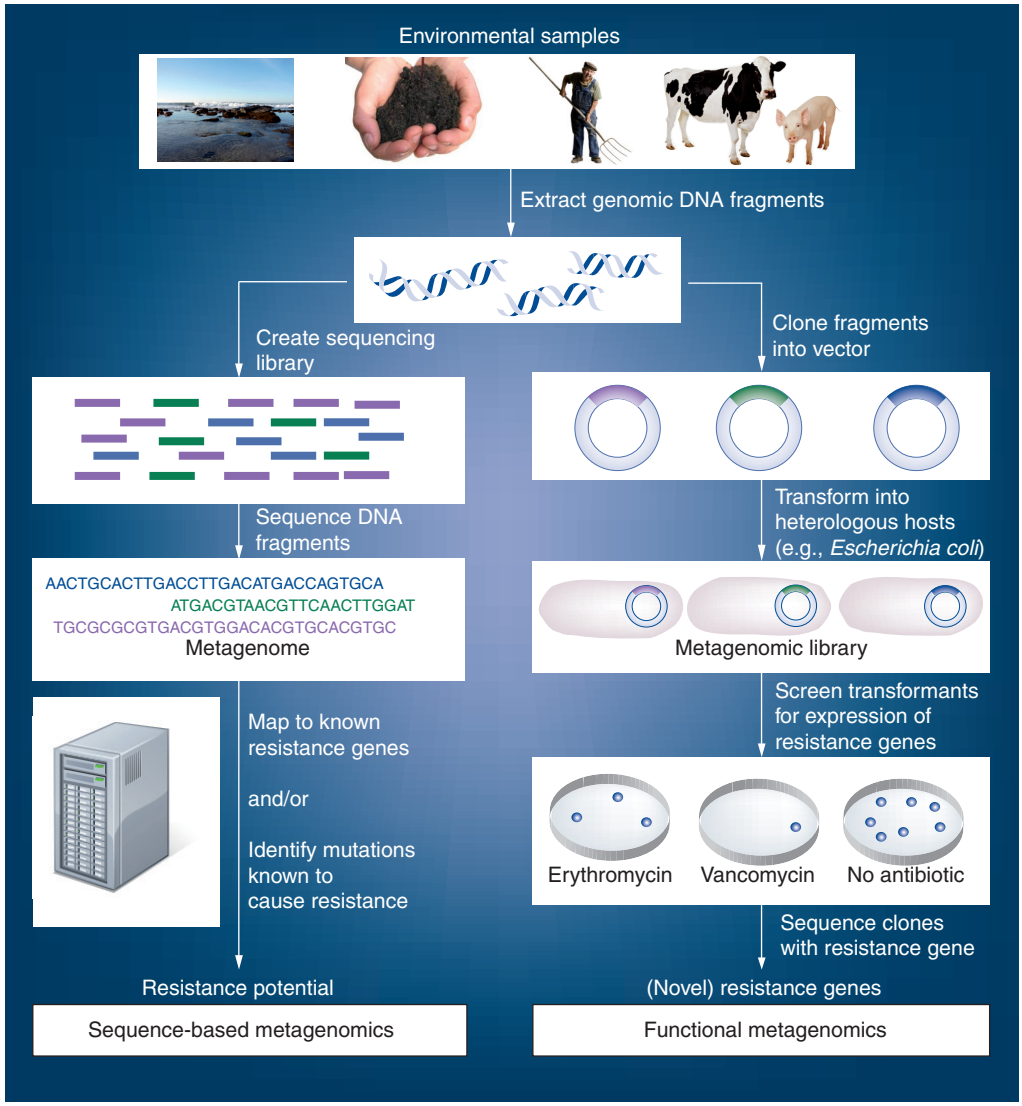

Figure 3. Metagenomic analysis of antibiotic resistance in microbial communities.

analysis tools (Figure 4) and sequence databases, such as IMG [32], MG-RAST [33], CAMERA [34] and the Sequence Read Archive [35]. Unfortunately, there is not a single authoritative source for all of the raw metagenomic sequence data, and the quality and descriptions of the data varies between databases and between datasets.

Antibiotic resistance in microbial communities

Antibiotics are widely used in the clinical setting to treat a range of diseases, and recent studies are directed at understanding the impacts of those treatments on the human microbiota. In addition, antibiotics are used in agricultural settings, and the impact of those treatments on human health is controversial. In the next sections we discuss the identification of antibiotic resistance genes and their impact on human health; the isolation of antibiotic resistance genes from the environment and the implications for the evolution of new resistance mechanisms; and how the agricultural use of antibiotics may influence both human health and resistance evolution.

Antibiotic resistance in human-associated microbes

It is estimated that there are ten-times as many microbes in and on any given human as there are human cells in that person's body, and 100-times as many unique genes than in our own genome [36]. The majority of these microbes reside in the gut, with an estimated 800–1000 different bacterial species in the gut community [37]. Those microbes are crucial for human life. However, approximately 80% of the gut microbiota have not yet been cultured [38].

In recent years, large-scale projects have been started as part of the International Human Microbiome Consortium to investigate the human microbiota. The Human Microbiome Project (HMP), an initiative of the NIH, was launched in 2008 with the mission to sequence, characterize, and analyze as much of the human microbiota as possible in 5 years [39,40]. Part of the HMP is to learn about microbes through metagenomics by sampling from five body sites: the mouth, nasal cavity, skin, gut and (in women) vagina of healthy donors. The HMP also funded 15 demonstration projects for 1 year to look at the microbiome in correlation to human health and awarded continued funding to eight of the projects. In addition to the HMP, the Metagenomics of the Human Intestinal Tract (MetaHIT) project [41] applies metagenomics to the study of the human intestinal tract of European and Asian populations.

A recent MetaHIT metagenomic study of 124 individuals suggests the existence of a common core human gut microbiome [41], but this core may exist more at the level of shared functional genes rather than shared taxa [42]. An open question in microbial ecology is whether selection operates at the level of taxa or the level of the functions that those taxa perform. Despite the core microbiome, there is a subject-to-subject variability in the composition of the gut microbiota among humans. Numerous internal and external factors, including diet, geography, host physiology, disease state, and features of the gut itself contribute to the community composition of the gut microbiota, and the HMP and related studies will significantly extend our understanding of these relationships [36,43,44].

Humans have directly exposed their bodies to antibiotics in medicines and indirectly through agriculture and cleaning or beauty products. On average, an adult in the USA makes two outpatient visits per year and 15.3% of these visits result in the prescription of an antibiotic [45]. Antibiotics prescribed during clinical visits may have long-term consequences on the human microbiota; community level studies demonstrated that the gut microbiota recovery is incomplete after repeated antibiotic perturbation and that treatment might cause a shift to a different, but stable community [46].

Antibiotic-resistant strains persist in the human host environment in the absence of selective pressure [4,47]. For example, Sommer *et al.* characterized the functional resistance reservoir in two unrelated healthy individuals who had not been treated with antibiotics for at least 1 year [48]. The

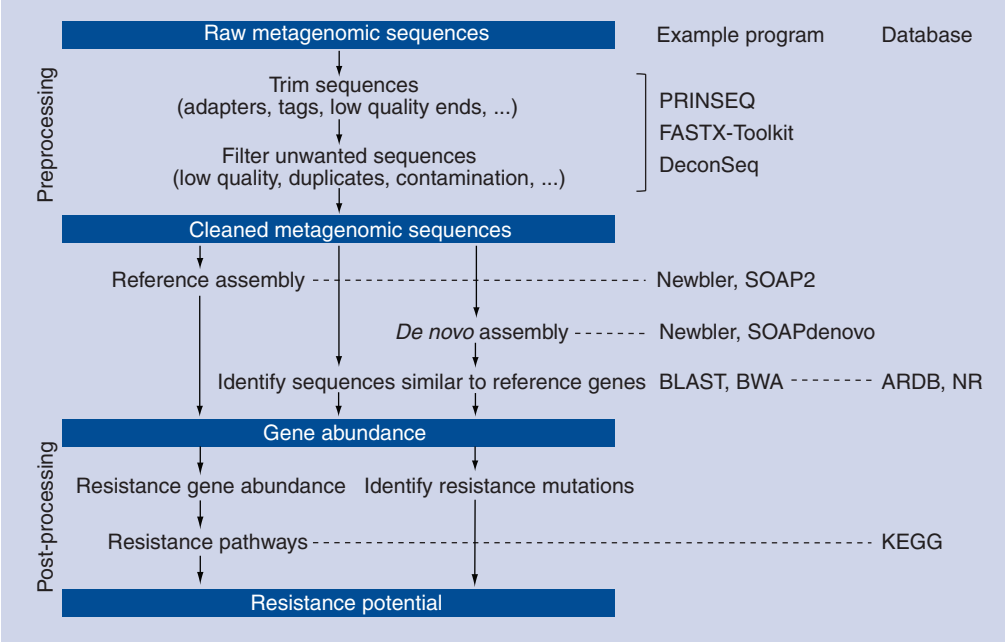

Figure 4. A proposed bioinformatics methodology to identify antibiotic resistance from sequence-based metagenomes. The methodology includes preprocessing steps, where a number of freely available tools can be applied, an analytical step where the sample is compared to databases such as the NCBI NR or the ARDB, and post-processing steps to identify the resistance potential. We encourage the reader to consult online resources, such as SEQanswers [105] and BioStar [106], for up-to-date information of current programs and databases.
ARDB: Antibiotic resistance database; NR: Nonredundant protein database.

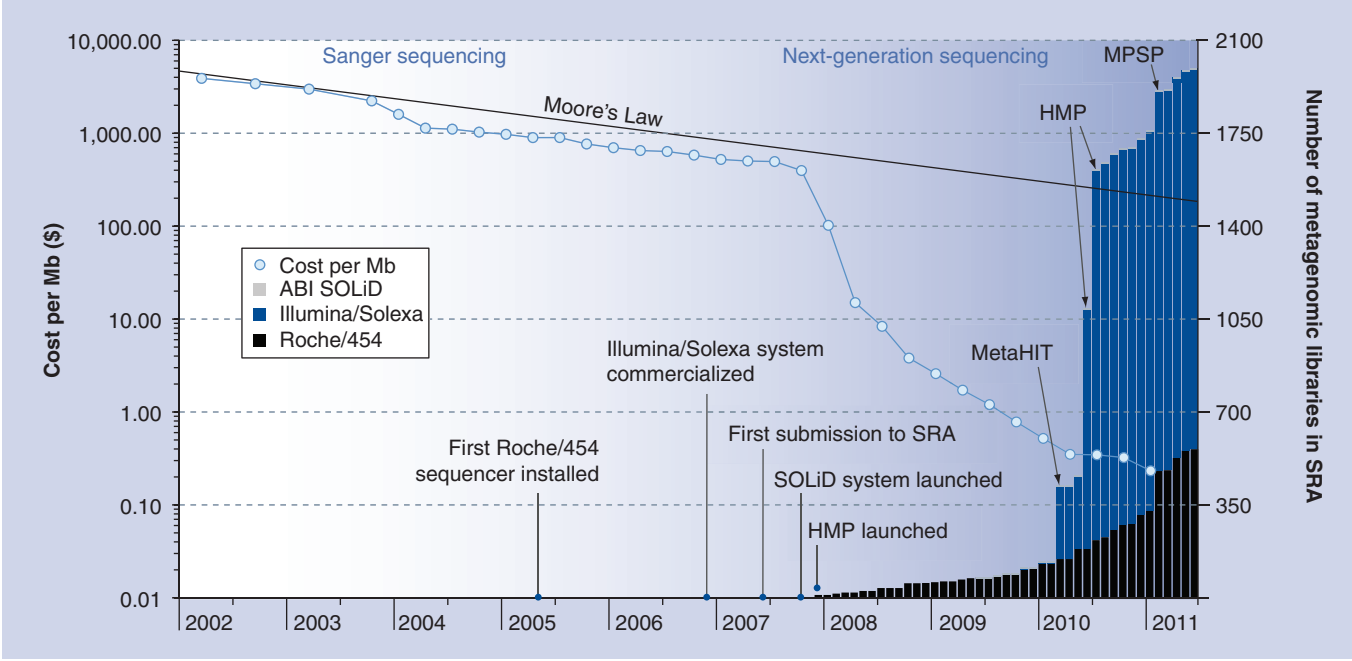

Figure 5. Overview of sequencing cost and number of metagenomic libraries submitted to the international Sequence Read Archive. The cost of sequencing is based on data provided by the US National Human Genome Research Institute [107], illustrating the more than logarithmic decrease and sudden change when the sequencing centers transitioned to next-generation sequencing technologies. The cost of sequencing is compared to Moore's Law, which describes a long-term trend in the computer hardware industry that involves the doubling of computing power every 2 years (here halving of sequencing cost every 2 years). The stacked bars show the number of metagenomic libraries that were submitted to the Sequence Read Archive (as of 1 July 2011) with a specified study type of 'Metagenomics' and a library strategy of 'WGS' (whole genome shotgun sequencing). The sequencing technologies used to generate the metagenomic libraries are marked as shown in the legend. As of July 2011, only one metagenomic library in this category was generated using the SOLiD system. Major milestones in next-generation sequencing and major submissions (>100 libraries) are highlighted. HMP: Human Microbiome Project; MetaHIT: Metagenomics of the Human Intestinal Tract; MPSP: Marine Phage Sequencing Project; SRA: Sequence Read Archive.

subject's microbiota was analyzed using functional screening of metagenomic DNA and genomic DNA from 572 bacterial isolates. The sequencing of inserted clones conferring resistance to 13 different antibiotics revealed 95 unique inserts that were evolutionarily distant from known resistance genes. This diverse gene pool of resistance genes in the commensal microbiota of healthy individuals could potentially lead to the emergence of new resistant pathogenic strains. It may also suggest that barriers exist to lateral gene transfer between the bacteria with the novel resistance genes and readily cultured human pathogens [49]. We do not know where the resistance mechanisms come from, but they are easily acquired. For example, a study with a newborn showed that methicilin and flouroquinolone resistance genes and multidrug efflux pumps could be found in the meconium 3 days after birth [50]. At day 6, teicoplanin resistance genes were found in the infant fecal sample. In this particular individual, a fever at day 92 was followed by the presence of β-lactamase genes and a reduction in bacterial load in the stool, even though antibiotics were not reported to be used at that time.

Similar to the human gut microbiata, an important attribute of the oral microbiota is its ability to act as a reservoir of antibiotic resistant organisms. Oral bacteria can easily reach other body sites by swallowing or via the bloodstream and spread to other individuals by, for example, coughing and kissing. Therefore, resistant oral bacteria have the opportunity for rapid dissemination.

A novel tetracycline resistance determinant, *tet37*, was identified by Diaz-Torres *et al.* who constructed metagenomic libraries from the microbiota of the human oral cavity [24], and then 3 years later, they applied functional metagenomics to identify genes encoding antibiotic resistance in the oral microbiota of 60 adult humans [51]. Clones resistant to tetracycline and amoxycillin were detected in each library, and the former was the most common resistance in each library. They did not report on their patient's antibiotic usage, and so it is not known how that affected their experiments.

Our oral and fecal microbiota are distinctly different, both in the organisms present and the resistance mechanisms found in those organisms.

Seville *et al*. investigated the presence and prevalence of various tetracycline and erythromycin resistance genes in the metagenomic DNA isolated from human oral and fecal samples from six different European countries [52]. Although the subjects did not receive antibiotics for 3 months, the profile of resistance genes detected in the fecal metagenome differed considerably from those detected in the oral samples, again suggesting that exposure to antibiotics has long term affects on the microbiota.

The fate of antibiotics used in human medicine is largely unknown. After secretion from the human, some antibiotics are broken down by resistance, some are light, or temperature labile, while others may exist for a long time in the environment. As we shall see later, antibiotics in the environment may promote the development of novel mechanisms of antibiotic resistance.

Antibiotic resistance in soil microbial communities

There are as many microorganisms in 1g of soil as there are humans on the entire planet [53], and much less than 1% of those microbes are readily culturable by current methods [54,55]. Soil is most likely the ecosystem where antibiotic synthesis originally evolved [56] and soils from diverse locations around the world such as forest, prairie, agriculture and urban have been explored to develop new clinical and medicinal applications. As a result, over 80% of antibiotics in clinical use today originated from soil bacteria, either directly as natural products or as their semi-synthetic derivatives [57].

The high density of antibiotic-producing bacteria makes soil a likely source of diverse antibiotic resistance determinants [20,23,58,59]. Molecular resistance mechanisms between clinical pathogens and the common soil bacterium *Streptomyces* were first shown to be similar in 1973 [60]. Since then, numerous parallels have been identified between soil microorganisms and clinically important strains, and the abundance of pathogens that can survive in soil results in a potent mixture that can give rise to the emergence of antibiotic resistance in the clinical setting. In recent years, metagenomic approaches have been implemented to characterize the diversity and prevalence of resistance in soil bacteria [61].

The metagenomic studies of soil environments show evidence that multiple diverse mechanisms for resistance are associated with microbes from different soil samples (Table 1). These studies also suggested that soil microbes are resistant to most antibiotics, and the broad activity of these resistance genes might afford protection against newly developed antibiotics.

It is assumed that many soil bacteria are naturally resistant to antibiotics such as β-lactams [22,62]. Allen *et al*. identified genes that mediate resistance to β-lactam antibiotics from remote Alaskan soil with no known exposure to anthropogenically derived antibiotics [22]. They performed functional metagenomics to allow comparison of antibiotic resistance between the Alaskan soil and soils subjected to human activity, and to search for florfenicol resistance genes [59]. Torres-Cortés *et al*. applied functional metagenomics to soil samples and identified a gene belonging to a new type of reductase conferring resistance to trimethoprim, a synthetic antibiotic that interferes with the production of tetrahydrofolic acid [63].

Antibiotic resistance is common in soil bacteria. Nine clones expressing antibiotic resistance in *E. coli* to five different aminoglycoside antibiotics and one clone expressing tetracycline resistance were identified in a metagenomic library from soil samples [20]. These resistance proteins were less than 60% identical to previously published sequences, suggesting that soil microorganisms harbor more genetic diversity than previously assumed [63]. More recently, 446,000 clones comprising a soil-derived metagenomic library were screened for genes conferring resistance to tetracycline, β-lactams, or aminoglycoside antibiotics, which resulted in the identification of 13 different antibiotic-resistant clones [23]. The low level of sequence similarity from these clones suggests that these proteins may play a different role in natural environments. For example, it was proposed that antibiotics could, in reality, be signal molecules that help shape the structure of microbial communities [64–66]. Alternatively, resistance may be associated with adaption to, and survival in, nutrient-poor environments, and some bacteria can use antibiotics as a food source, presumably via resistance mechanisms they have developed [67]. Further studies on a more diverse subset of strains, especially slow-growing strains and those difficult to culture will be important to clarify the role of resistance in bacteria.

Antibiotic resistance associated with wastewater treatment plants

Wastewater treatment plants are interfaces between different environments and, therefore, provide an opportunity for mobile elements (including resistance) to mix between pathogens, opportunistic pathogens, and environmental

Table 1. Summary of resistance genes identified through functional metagenomics.

Environment	Number of clones screened (total library size)	Number of resistant clones	Resistant to (predicted mechanisms)	Ref.
Soil (plano silt loam)	1,158,000 (4.1 Gb)	9 1	Aminoglycoside (inactivation) Tetracycline (efflux)	[20]
Soil (apple orchard)	446,000 (13 Gb)	3 1 9	Aminoglycoside (inactivation) Tetracycline (efflux) β-lactam (inactivation)	[23]
Soil (Alaskan)	714,000 (12.4 Gb)	14[†]	β-lactam (inactivation)	[22]
Soil (Alaskan)	-[‡] (13.2 Gb)	1	Amphenicol (efflux)	[59]
Soil (loamy)	550,000 (3.6 Gb)	2 3 2 4	Aminoglycoside (inactivation) β-lactam (inactivation) Amphenicol (efflux) Antifolate (inactivation)	[63]
Activated sludge	- (1.8 Gb) 1161 (1.2 Mb)	1 1 7 0[§]	Aminoglycoside (inactivation) β-lactam (inactivation) Amphenicol (inactivation) -	[69]
Activated sludge	96,000 (3.2 Gb)	3[¶]	Glycopeptide (inactivation)	[25]
Human (fecal, saliva)	-[#] (9.3 Gb)	356 2408 12 1330 191	Aminoglycoside Amino acid derivative Amphenicol β-lactam Tetracycline	[48]
Human (plaque, saliva)	450[††] (-)	18	Tetracycline	[24]
Human (plaque, saliva)	1860[††] (-)	14 32 58	Aminoglycoside β-lactam Tetracycline	[51]
Insect (midgut disect)	38,500 (0.3 Gb)	2 3[‡‡] 1[‡‡]	Macrolide (efflux) β-lactam (inactivation) Amphenicol	[29]
Organic pig (fecal)	9000 (0.1 Gb)	10	Tetracycline	[87]

[†]Resistance genes could only be identified in 13 clones.
[‡]Library from [22] used.
[§]Phage metagenomic library.
[¶]Two of three resistant clones were assumed to be from the same region due to identical sequences.
[#]Insert length between 1000 and 3000 bp.
[††]Insert length between 800 and 3000 bp.
[‡‡]One clone was resistant to all three antibiotic classes in a secondary screen.

bacteria [68]. The presence of antibiotics in sewage selects for resistance markers that are able to spread through the microbial community and as a result, antibiotic-resistant bacteria can potentially disseminate their resistance genes widely among members of the endogenous microbial community (Figure 6). The sludge products of urban and rural wastewater treatment plants are increasingly used to fertilize agricultural crops, dispersing unknown amounts of resistance genes and antibiotics that withstand standard sewage treatment.

The level of antibiotic resistance in activated sludge is not known, and the few studies to date have been inconclusive. The activated sludge process may promote cellular interactions among all the diverse microorganisms in the sewage, but it is also a highly competitive environment where the fraction of pathogens decreases due to competition with other microbes or through predation. A function- and sequence-based metagenomic approach was used to identify antibiotic resistance determinants carried on

bacterial chromosomes, plasmids or viruses within activated sludge (Table 1) [69]. Functional screening of the viral metagenomic library did not yield any antibiotic resistant clones, and therefore, a sequence-based approach was employed that identified six clone inserts with homology to known resistance genes. In contrast to some of the studies we have discussed, the degree of similarity between the plasmid- or phage-derived antibiotic resistance determinants and the reference sequences was greater than that for other genes that were tested. However, none of the sequenced clones conferred their predicted antibiotic resistance in *E. coli*. There are a number of reasons that the genes may not confer resistance to *E. coli*, including the incorrect expression in that strain, or the incomplete cloning of the sequence. In another study, activated sludge used to treat industrial wastewater polluted with phenolic compounds, was screened for bleomycin resistance genes using a metagenomics approach and two novel genes were identified [25].

A sequence-based metagenomic approach of wastewater using Roche/454 pyrosequencing showed a high diversity of plasmids and resistance genes in that sample [70]. The metagenomic sequences representing resistance genes, especially from β-lactamase protein families, were identified by searches against reference databases with known antibiotic resistance genes and by screening for particular motifs in the protein sequences. These motifs and sequences were similar to enzymes that were previously identified in pathogenic bacteria isolated from hospital patients with diverse infections, but were not tested for activity.

High levels of antibiotic resistance genes have also been found in bacteria that live in river sediments downstream from a wastewater treatment plant using sequence-based metagenomics [71]. The resistance genes made up almost 2% of the DNA samples taken. Clearly, the survival of antibiotics and antibiotic resistance genes through the sewage treatment process and in the aquatic environment merits further study, perhaps with comparisons between areas where different antibiotics are routinely used in the clinical setting.

Antibiotic resistance in marine microbial communities

Antibiotic resistant bacteria have been found widely in aquatic environments [72,73]. As we discussed earlier for terrestrial environments, resistant organisms in marine environments can occur following the introduction of antibiotics from agricultural runoff or wastewater treatment plants. Aquaculture farms are a significant source of antibiotics contaminating marine environments, because antibiotics are added to overcome the diseases that predominate in the high animal density cages. There is evidence that antibiotic resistance can also occur in marine environments without the addition of antibiotic contamination. For instance, the same resistance genes found in clinical human pathogens have been reported among pristine ecosystems without a history of antibiotic contamination [74,75]. For example, a sequence-based metagenomic study of the microbial community associated with the coral *Porites astreoides* showed the presence of fluoroquinolone-resistance genes [76]. The authors proposed that the coral harbors

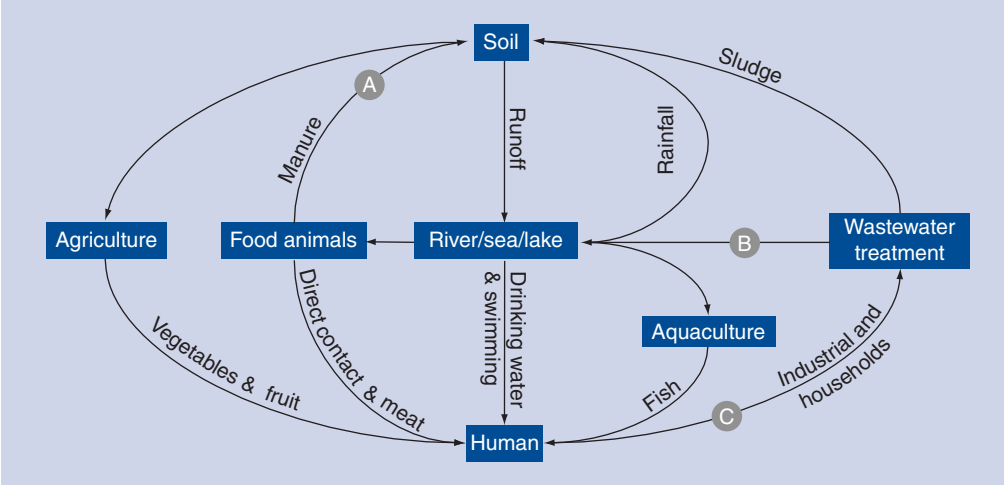

Figure 6. Potential antibiotic resistance gene dissemination. The arrows indicate possible points of dissemination among different environments. Supporting metagenomic studies are marked as follows: **(A)** [82], **(B)** [71] and **(C)** [48,52].

specialized microbiota that may protect the coral from pathogens by producing antibiotics; however, there was no evidence of fluoroquinolone production in the metagenomes and there was no evidence of a human-derived source of fluoroquinolones into the environment.

Animal- & agriculture-related resistance

Only approximately half of antibiotics produced each year are used for human health. The other half is used for industrial farm animal production. Some are used for therapeutics, but mostly the antibiotics are added at subtherapeutic levels to feedstuffs where they act as growth promoters [77]. The use of subtherapeutic doses of antibiotics is extremely controversial – it helps to keep the costs of meat and poultry in the USA low, perhaps reduces the overall number of animals that need to be reared, reduces fat in the meat, and reduces environmental impacts, but at the cost of providing an enormous source for the evolution of antibiotic resistance elements that may affect human health.

Antibiotics were first approved for the use as growth promoters in food animal production in the early 1950s, and since then the extensive use of subtherapeutic levels of antibiotics has been shown to promote antibiotic-resistance [78,79] and animal feed has been found to contain antibiotic resistance genes [80]. In 1986, Sweden was the first country to ban the use of antibiotic growth promoters in food animal production, and 20 years later the EU banned the feeding of antibiotics to livestock for growth promotion (although they are still used therapeutically). In the USA, cattle are usually treated with monensin, which is not directly related to antibiotics used in human therapeutics, while poultry are often fed bacitracin and tetracycline, which are both used in human medicine.

Because the issues surrounding the agricultural use of antibiotics are complex, and the spread of resistance mechanisms might potentially affect human health, metagenomics has been used to try to understand the flow of resistance genes associated with this source of antibiotics. One of the problems with agricultural antibiotic use is that the antibiotic is not restricted solely to the animal or farm where it is applied: residues from farms can contain antibiotic-resistance genes that may contaminate natural environments, wastewater treatment plants, and as we have seen, impact environmental microbiota [81]. The fecal component of cattle manure carries a large community of bacteria and is thus a natural vehicle for transmission of bacteria into the environment and onto other animals [82]. In particular, a variety of insects feed on cow manure, and insect guts are environmental reservoirs of antibiotic resistant bacteria with the potential for dissemination of resistance genes [29]. Manure applied to agricultural fields can also contaminate the surface of food crops, including vegetables and fruits [83] and has led to many of the well publicized outbreaks of food-borne illness in the USA and elsewhere. In addition, antibiotic-resistance genes can be aerosolized and carried away from the farm; they have been found downwind (but not upwind) of a large agricultural facility [84]. A sequence-based metagenomic study of the chicken cecum microbiome showed that resistance to tetracyclines and fluoroquinolones were dominating that environment, both classes of antibiotics that are routinely used in poultry production [85]. A further comparison of the virulence-associated sequences showed that the chicken cecum was more similar to the microbiome of bovine rumen than to that of mouse cecum or human fecal samples [85], perhaps not surprising given the physiological differences in the intestine, rumen and cecum. Other studies suggest that the use of antibiotics in agriculture may have resulted in the spread of strains, such as vancomycin-resistant enterococci in both farm animals exposed to antibiotics and humans in contact with the animals [78,86], as well as the maintenance of antibiotic-resistance genes in apparently antibiotic-free animals [87].

Detailed discussion of the problems and challenges associated with agricultural antibiotic use can be found at the Pew Charitable Trusts website [103], and at the Animal Health Institute's website [104].

Ongoing challenges for detecting antibiotic resistance in environmental samples

As we have discussed, metagenomics can be used to identify antibiotic resistance genes in the environment, and has increased our understanding of the sources and roles of these genes in nature. However, there are problems associated with metagenomics, and in the next sections we discuss some of those limitations.

Functional metagenomics

Functional screening, where fragments are cloned and expressed, may be hampered by the clone and insert size compared to the total metagenome size. Antibiotic resistance can be

encoded by multiple genes that are required to work together (e.g., vancomycin resistance), by a single gene (e.g., *bla*, the gene encoding β-lactamase), or by a point mutation in housekeeping genes (e.g., *gyrA,* the gene that encodes DNA gyrase). Each of these results in difficulties and challenges for metagenomic screens.

The results of functional metagenomics are dependent upon each gene's ability to be expressed in surrogate hosts, typically *E. coli* [88]. Resistance genes are regulated by genetic elements that may not be recognized by the surrogate host's gene expression machinery, the codon usage may not be appropriate for expression, post-translational modifications may be missing, or the expressed protein may not fold correctly. Heterologous gene expression can also result in false positives since the foreign gene may interact in novel ways with the cellular machinery.

The range of media types, antibiotic concentrations and incubation methods used to measure resistance levels make it difficult to compare results between environments [18]. Diaz-Torres *et al*., for example, found that there are problems with the expression of certain tetracycline resistance genes found in the human oral microbiome when *E. coli* was used as a host [51].

In addition to heterologous gene expression issues, genes and their encoded proteins are adapted to different environmental conditions that might preclude efficient enzyme activity at 37°C in standard laboratory media. For example, genes extracted from Alaskan soil microbes that live at temperatures much lower than 30°C [22,59], and insect midguts, where microbes live in an environment with a pH of 12.4 [29]. It is possible that many of the genes in functional metagenome libraries are not expressed in *E. coli*, resulting in an underestimate of the frequency of resistance determinants in environmental samples. Some resistance genes have been found using a wider range of expression systems and hosts, but global sequencing approaches indicate a large discrepancy between predicted and detected resistance genes [89]. The limitations of *E. coli*-based screening call for the broader use of a wider range of hosts including not only Gram-negative, but also Gram-positive host species.

Another study was not able to identify streptomycin-resistant clones in functional screens, despite their presence in the metagenomic library verified using PCR and culturable bacteria [23]. In addition to unidentified resistance genes, a large number of resistant clones may be false positives [63].

Sequence-based metagenomics

The biggest challenge with sequence-based metagenomics is the large number of sequences that show no significant similarity to previously sequenced genes or organisms; without known reference sequences, resistance genes cannot be easily identified in the metagenomes. The strong selection for antibiotic resistance alleles results in convergent evolution – the adaption of very different genes to perform the same function [16,90,91]. We noted above that many resistance genes identified in functional screens have low similarity to known genes, but with sequence-based approaches we are generally limited to only identify things we already know.

The current sequence-based metagenomic approaches need to be evaluated based on the complexity of technical procedures, robustness, accuracy and cost. The preparation of a sample library requires multiple molecular biology steps and, depending on the technology, up to 4 days to complete. The range of data volumes leads to processing times from a few minutes to multiple hours, emphasizing the need for sufficient computation power. The data analysis requires both expertise in bioinformatics and a more advanced informatics infrastructure.

High-throughput sequencing technologies generate data that currently challenge data storage, management and processing, demanding access to supercomputing resources or cloud-based computing services for efficient handling. Advances are needed in data transfer and management, standardization of data formats, and integration of different types of data [92]. The amount of data is even challenging national data warehouses, such as the Sequence Read Archive, which announced that it will limit data archiving to a specific subset of next-generation sequence data starting from October 2011.

The sequence-based identification of a resistance gene requires separate functional confirmation. The metagenomic sequences only suggest the presence of an enzyme that may encode antibiotic resistance, but do not necessarily confirm that a gene is functionally expressed or that it does not encode an alternative function in its host. With the advancement of mRNA extraction from environmental samples, metatranscriptomics may become the main method for the detection of functional resistance genes [93].

Whether the methodology employed in microbiota characterizations are faithfully reproducing the community composition, and not distorting it due to experimental bias or sequencing

artifacts is still an open question. However, as sequencing becomes cheaper, researchers will be able to sequence deeper and at multiple time points to address possible biases, and to answer the important questions about the sources, evolution, and effects of antibiotic resistance genes that we have touched on here.

Conclusion

We are dependent on antibiotics for the treatment of infectious diseases and they are critical for the success of advanced surgical procedures, such as organ and prosthetic transplants. Antibiotic-resistance mechanisms create an enormous clinical and financial burden on healthcare systems worldwide. Despite the problem of antibiotic resistance in infectious bacteria, little is known about the diversity, distribution and origins of resistance genes, especially for the unculturable majority of environmental bacteria.

There exists high-level resistance both to antibiotics that have for decades served as gold standard treatments and to those only recently approved for human use. The study of the environmental resistance reservoir using metagenomic approaches will provide an early warning system for future clinically relevant antibiotic-resistance mechanisms. Knowledge of such natural variations will complement studies on clinical isolates to guide the rational development of next-generation antibiotics that will be active against resistant strains.

Functional and sequence-based metagenomics have been used for the discovery of novel resistance determinants and the improved understanding of antibiotic resistance mechanisms. Metagenomic sequence data can be used to generate sample-specific and temporal antibiotic resistance profiles to facilitate an understanding of the ecology of the microbial communities in an environment as well as the epidemiology of antibiotic resistance gene transport between and among environments.

Traditional approaches to antibiotic resistance typically concentrate on human pathogens. There is a clear need to expand the focus to include nonpathogenic bacteria in antibiotic research. This may allow researchers to predict resistance before it emerges clinically; to develop diagnostic techniques, and to build new therapeutic strategies to counteract resistance before it emerges in human pathogens. With respect to functional metagenomics, we need to develop and apply new approaches to cultivate the previously uncultivated and rare members of the microbial communities to assign functions to the vast number of unknown or hypothetical genes, and to develop novel genetic systems that allow screening of the vast array of microbes on earth to identify antibiotic-resistance genes.

Sequence-based metagenomics allows the comparison of microbial communities from different hosts to investigate differences in the response to antibiotics and to select the most favorable antibiotic to reduce side effects for certain hosts.

The transition of next-generation sequencing into clinical diagnostics is in the early stages of development in large reference laboratories and is being leveraged for applications that require large amounts of sequence information. Sequence-based metagenomic data has the ability to combine antibiotic-resistance gene abundance data with community composition and metabolic pathway information to provide a more complete profile of specific samples. This information is of great importance for the implementation of rational administration guidelines for antibiotic therapies. We may also use these techniques to learn how human-associated microbes can be manipulated by antibiotics or probiotics to reduce our dependency on antibiotics, provide alternatives to existing antibiotics, and extend the lifetime of current and new antibiotics.

Many questions remain: the roles of the environmental reservoirs in clinical resistance development are still hypothetical; we have little or no evidence that any of the putative resistance genes identified in the environmental studies have been mobilized into pathogenic bacteria and expressed as resistance phenotypes; we do not know how to rapidly identify new resistance genes; and functional approaches are still relatively low throughput.

Future perspective

The application of metagenomics will not only facilitate future identification of novel resistance genes, but will be used for the prediction of future evolution of antibiotic resistance and will enable further studies on genetic elements participating in resistance gene transfer.

Within the next few years, we will see the application of metagenomics or sequence-based technologies in almost every part of life and biomedical sciences, in particular in clinical diagnostic settings. To date, most sequence-based metagenomic studies are performed in research laboratories using the Roche/454 and Illumina systems (Figure 5). With the promises of

third-generation sequencing technologies, such as higher throughput (amounts of DNA that can be processed per unit time) with increased accuracy, longer read lengths, lower cost, smaller amounts of starting material and shorter turnaround times (time to result), these technologies are anticipated to transition into clinical-diagnostics use over the next several years. In June 2011, the US FDA held a public meeting to discuss the use of ultra high-throughput sequencing for clinical diagnostic applications. In addition to sequencing technologies, the bioinformatics analysis of the sequence data was a major focus of the meeting. The large amounts of sequence data generated with next-generation sequencing technologies pose a bioinformatics challenge for the clinical laboratory to provide data processing and interpretation to the clinicians. New algorithms, visualization tools, and data abstractions will be needed to cope with the challenges presented by this data.

High-throughput sequencing allows for global analysis of commensal populations and potential identification of distinct signatures in the human microbiome. As sequencing becomes less expensive, repeated sampling of the metagenome of a microbial community can be used to evaluate changes in the community over time, and whole-body metagenome approaches can be applied in large-scale research and clinical studies. For example, urine and feces provide two windows that reflect the health status of the human body, and sequencing the microbiomes associated with these samples will provide a snapshot of overall health. We will also move towards personalized prescriptions, a subset of the genome-inspired personalized medicine. Antibiotic treatment of high-risk patients will not stop, but metagenomics will help us understand the effects of different classes of antibiotics on the composition and function of commensal and pathogenic microbial populations. These insights will allow the switch from broad- to narrow-spectrum antibiotics, antibiotics that target very specific pathogens, and very specific courses of treatment instead of the standard 7–10 days. In addition, a new category of therapeutics will be developed that target the microbiota and modulate microbe–microbe and microbe–human interactions. These therapeutics will allow us to alter the composition of the microbial community, endowing it with new functions, and use the sequences of the microbiota to diagnose disease. In the future, this might be integrated into daily life; for example, DNA sequencers in toilet bowls that automatically follow changes of the human microbiome and suggest appropriate antibiotic treatment.

Acknowledgements

The authors would like to thank Linda Wegley, Molly Schmid and Anca Segall for helpful comments, suggestions and thought provoking discussions.

Financial & competing interests disclosure

This work was funded by NSF grants DBI:0850356 from the Division of Biological Infrastructure and DEB:1046413 from the Division of Environmental Biology (both to R Edwards). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Mechanisms of antibiotic resistance

- Resistance to antibiotics can be caused by four general mechanisms (inactivation, alteration of the target, circumvention of the target pathway or efflux of the antibiotic) and bacteria can develop resistance by mutating existing genes, or by acquiring new genes from other strains or species.

Detection of antibiotic resistance

- Antibiotic resistance is a highly selectable phenotype and can be detected using growth inhibition assays; however, culture-based approaches can take days to weeks for slow-growing bacteria and only work for a fraction of microbes.
- Antibiotic resistance genes were typically isolated by cloning from cultured bacteria or by PCR amplification. Those methods missed potential antibiotic resistance reservoirs because of uncultivable bacteria or dependence on known resistance genes.
- Metagenomics overcomes the limitations of culture-dependent techniques and is a powerful tool to identify novel resistance genes and to describe the presence or absence of genes or genetic variations responsible for antibiotic resistance.

Culture-independent study of resistance through metagenomics

- Functional metagenomics involves cloning and expression of DNA in a surrogate host with coupled activity-based screening.
- Sequence-based metagenomics involves extracting and random sequencing of DNA directly from the environment. The metagenomic sequences are then compared to known sequences to identify resistance genes and/or mutations.

Executive summary (cont.)

Antibiotic resistance in human-associated microbes

- It is estimated that there are ten-times as many microbes in and on any given human as there are cells in that person's body and the majority of those have not yet been cultured. Large-scale sequencing projects such as the HMP and MetaHIT have been started as part of the International Human Microbiome Consortium to investigate the human microbiota.
- Antibiotics may have long-term consequences on the human microbiota, which cannot fully recover after repeated antibiotic perturbation, and might cause a shift to a different, but stable community.
- Our oral and fecal microbiota are distinctly different, both in the organisms present and the resistance mechanisms in those organisms.

Antibiotic resistance in soil microbial communities

- There are an estimated 10^9 microorganisms per gram of soil and less than 1% of those microbes are readily culturable by current methods. The high density of antibiotic-producing bacteria makes soil a reservoir for antibiotic resistance.
- Metagenomic studies of soil environments identified novel and known genes that mediate resistance to different classes of antibiotics, including soil from remote locations with no known exposure to antibiotic drugs. The resistance proteins had low similarity to previously published sequences suggesting that soil microorganisms harbor more genetic diversity than previously assumed.

Antibiotic resistance associated with wastewater treatment plants

- Wastewater treatment plants are interfaces between different environments and provide an opportunity for resistance to mix between pathogens, opportunistic pathogens, and environmental bacteria. The sludge products are increasingly used to fertilize agricultural crops, dispersing unknown amounts of resistance genes and antibiotics.
- Activated sludge showed evidence for a high diversity of resistance genes and allowed the identification of novel resistance genes.
- The survival of antibiotic-resistance genes through the sewage treatment process and in the aquatic environment merits further study.

Antibiotic resistance in marine microbial communities

- Antibiotic-resistant bacteria have been found widely in aquatic environments and resistance genes found in clinical human pathogens have been identified in marine microbes.

Animal- & agriculture-related resistance

- Antibiotics are extensively used for animal farming and for agricultural purposes and may have effects on the selection of resistance.
- Residues from farms can contain antibiotic resistance genes that may impact environmental microbiota.

Ongoing challenges for detecting antibiotic resistance in environmental samples

- The results of functional metagenomics are dependent upon each gene's ability to be expressed in surrogate hosts. The limitations of *Escherichia coli*-based screening most likely resulted in an underestimate of the frequency of resistance determinants in environmental samples. In addition, a range of media types, antibiotic concentrations and incubation methods make it difficult to compare results between environments or laboratories.
- The biggest challenge with sequence-based metagenomics is the large number of sequences that show no significant similarity as sequence-based approaches are generally limited to only identify things we already know. High-throughput sequencing technologies generate data that currently challenge data storage, management and processing, demanding access to supercomputing resources and expertise in bioinformatics.
- Sequence-based identification of a resistance gene requires separate functional confirmation; however, with the advancement of mRNA extraction from environmental samples, metatranscriptomics may become be the main method for the detection of functional resistance genes.

Conclusion

- Functional and sequence-based metagenomics have been used for the discovery of novel resistance determinants and the improved understanding of antibiotic-resistance mechanisms.
- Sequence-based metagenomic data has the ability to combine antibiotic resistance gene abundance data with community composition and metabolic pathway information to provide a more complex profile of specific samples.
- The study of the environmental resistance reservoir using metagenomic approaches could provide an early warning system for future clinically relevant antibiotic resistance mechanisms.

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