Next-generation approaches to understand and combat the antibiotic resistome

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Abstract

Antibiotic resistance is a natural feature of diverse microbial ecosystems. Although recent studies of the antibiotic resistome have highlighted barriers to the horizontal transfer of antibiotic resistance genes between habitats, the rapid global spread of genes that confer resistance to carbapenem, colistin and quinolone antibiotics illustrates the dire clinical and societal consequences of such events. Over time, the study of antibiotic resistance has grown from focusing on single pathogenic organisms in axenic culture to studying antibiotic resistance in pathogenic, commensal and environmental bacteria at the level of microbial communities. As the study of antibiotic resistance advances, it is important to incorporate this comprehensive approach to better inform global antibiotic resistance surveillance and antibiotic development. It is increasingly becoming apparent that although not all resistance genes are likely to geographically and phylogenetically disseminate, the threat presented by those that are is serious and warrants an interdisciplinary research focus. In this Review, we highlight seminal work in the resistome field, discuss recent advances in the studies of resistomes, and propose a resistome paradigm that can pave the way for the improved proactive identification and mitigation of emerging antibiotic resistance threats.

Antibiotics have revolutionized modern medicine and have saved millions of lives since their discovery at the beginning of the twentieth century. These crucial drugs are invaluable owing to their ability to kill or inhibit the growth of bacteria without damaging host cells and tissues[1,2] (BOX 1). Aside from directly curing deadly infectious diseases, antibiotics have

enabled physicians to pursue new treatments such as surgery, organ transplantation and cancer chemotherapy, which were previously infeasible or unsafe owing to the high collateral risk of infection[3]. Antibiotics remain one of the most commonly prescribed classes of drug, with more than 70 billion clinical doses administered globally in 2010, which represents a 36% increase in antibiotic consumption relative to 2000 (REF.[4]). Although the first antibiotics were synthetic compounds, most antibiotics are natural products or derivatives thereof[5]. Many antibiotics were recovered from cultured soil microorganisms, particularly those belonging to the *Streptomyces* genus[3]. As might be expected given their origins as natural products of bacterial secondary metabolism, many antibiotics can be detected at low levels in pristine environmental samples and have been found in samples that pre-date human antibiotic use[6,7].

As a result of the ubiquity of antibiotic production by environmental microorganisms, defence mechanisms against these antibiotics and thus resistance (BOX 1) also seem to be ancient. Sequencing of ancient environmental DNA preserved in 30,000-year-old permafrost sediments has detected genes that confer resistance to several classes of antibiotic[8], and the direct screening of bacteria cultured from a cave that had no anthropogenic contact for the past four million years revealed extensive resistance against 14 antibiotics[9]. Similarly, the host-associated microbiome of previously uncontacted indigenous people in South America was found to encode several antibiotic resistance genes[10]. This indicates that resistance to antibiotics far pre-dates the appropriation of these small molecules for human use.

The collection of resistance genes in a given environment, which is termed the 'resistome' (REFS[11,12]), encompasses both intrinsic and acquired resistance genes, as well as `proto-resistance genes` and silent or `cryptic resistance genes`. It is thought that many clinically relevant antibiotic resistance genes have their evolutionary origins in environmental microorganisms, which we define for the purposes of this article to comprise microorganisms that are not generally associated with humans. This hypothesis was pioneered by Benveniste and Davies[13], who, in 1973, noted biochemically identical resistance-conferring kanamycin acetyltransferases in environmental *Streptomyces* spp. and in clinical isolates of pathogenic *Escherichia coli*. Further evidence was provided by a recent functional screen of soil `metagenomes`, which revealed the presence of environmental antibiotic resistance genes that have >99% nucleotide identity to resistance genes in pathogenic isolates[14]. `Synteny` of these genes with other resistance genes and mobility elements suggests that they are likely candidates for past or future dissemination by `horizontal gene transfer` (FIG. 1).

The ancient origins of antibiotics and antibiotic resistance make it unsurprising that large-scale clinical and agricultural use of these compounds has selected for increasing resistance across the bacterial domain of life. Robust evidence for increasing environmental resistance exists in decades-old soil samples, in which increasing numbers of antibiotic resistance genes correlate with the industrial-scale production and use of these drugs[15]. The resulting deterioration of the efficacy of our antibacterial armamentarium has come with substantial costs that are expected to increase throughout the next decades. Worldwide, it has been estimated that at least 700,000 people die annually as a result of resistant infections, a figure that is predicted to increase to 10 million annual deaths by the year 2050 if the current

trajectory is not altered. Throughout the next few decades, the cumulative global cost of antibiotic resistance is predicted to exceed US$100 trillion[16].

The recent spread of *mcr1*, *kpc*, *bla*$_{NDM1}$, *qnr* and other plasmid-borne resistance determinants (BOX 2) emphasizes two themes. The first is that in the antibiotic 'arms race' against bacteria, humanity is rarely ahead. This was exemplified by the first widely used antibiotics, the sulfonamides, against which resistance was observed only 6 years following their mass introduction[17]. More recently, resistance to tigecycline was identified even before its approval for human use, let alone its introduction into the clinic[18]. Therefore, it is either a sign of hubris or a lack of understanding of evolutionary principles when new antibiotics are advertised as being resistance-proof. The second theme has been our relatively myopic (albeit understandable) focus on resistance in human pathogens. It is a tautology that antibiotics are most effective at treating human pathogens. They would not be antibiotics if they failed to effectively treat pathogens, but our emphasis on a few pathogen 'trees' initially resulted in the field of antibiotics being blind to the 'forest' of environmental bacteria. The targets of most antibiotics are both highly conserved and essential across bacterial taxa, and have therefore made excellent mediators of intra-kingdom communication and competition among bacteria throughout biological history[19]. When our view is expanded to include the vast diversity of non-pathogenic bacteria, it is not surprising to see that all genera of the bacterial kingdom have evolved resistance mechanisms that protect these vital processes.

In this Review, we examine these themes across three eras of antibiotic resistance research. The first era, which coincides with the golden age of antibiotics, was characterized by a focus on resistance in pathogens in the clinic, and by the ready supply of new antibiotics and the diverse antibiotic mechanisms of action. In this era, drawing inspiration from Koch, the research priority was on single resistance genes and enzymes primarily from pathogenic organisms in `axenic` culture. In the second era of antibiotic resistance studies, we became less restricted by `Koch's postulates` and shifted our attention to resistomes. The antibiotic resistance of both benign and pathogenic bacteria was studied at the microbial community level, and metagenomic approaches were incorporated to discover resistance determinants from diverse habitats. As we enter the next era of antibiotic resistance research, we should acknowledge the ubiquity of antibiotic resistance, and take steps to improve the development of new antibiotic therapies and the surveillance of emerging resistance threats, focusing on resistance genes that are the most likely candidates for horizontal transfer to pathogens. In the remainder of this Review, we provide brief overviews of these three eras of resistome studies, and we outline how distinct philosophies from each of these eras could be integrated to improve the translational effect of resistome research going forwards.

The golden age of antibiotics

Both Sir Alexander Fleming and Gerhard Domagk, who discovered penicillin and prontosil, respectively, warned of the danger of bacterial resistance developing following improper use of their antibiotics[20,21]. One of the first reports of antibiotic resistance was the finding that a cell-free extract from a coliform bacterium that was unaffected by penicillin was able to inactivate penicillin, which indicated enzymatic degradation of the drug[22]. In the following decades, the extensive use of penicillin in hospitals led to the development of widespread

resistance in *Staphylococcus aureus* and resulted in the 1953 penicillin-resistant *S. aureus* phage-type 80/81 pandemic, which represented the world's first superbug until the release of methicillin ended the threat[23]. Soon after, the emergence of multidrug-resistant strains of *Shigella* spp., following apparent horizontal gene transfer from faecal strains of *E. coli*, was reported. Remarkably, there was evidence that such transfers occurred in hospitalized patients during the course of their antibiotic treatment[24].

The first era of resistance studies was concomitant with the golden age of antibiotics. During this period, all of the major modern classes of antibiotic were discovered. Within years, many were rendered obsolete in at least some organisms owing to the acquisition and spread of resistance[17]. For a period of time, novel or previously known mechanisms of resistance that arose in new pathogens were commonly dealt with by developing and releasing a new antibiotic. Indeed, resistant bacteria were even welcomed by Domagk as an opportunity to increase the antibiotic market share[20]. During this era, resistance genes and bacteria were largely identified one gene and one strain at a time using culture-based methods. This low-throughput approach, combined with incompletely exhausted reserves of natural antibiotics, disguised the otherwise apparent threat that antibiotic-resistant bacteria were destined to become.

High-throughput resistance discovery

The second era of resistome studies has been characterized by a broader ecological view of resistance, which was enabled by several breakthrough technologies. In the past few decades, the development of the new ecological resistome philosophy that recognizes the environment as the plausible original source of resistance genes and as the reservoir of resistance gene diversity has been driven by the technical advances in molecular biology and nucleic acid sequencing.

Sequencing-based resistance discovery

The development of genomic techniques that enable the culture-independent study of diverse environmental microbial communities has been a crucial driver of the paradigm shift in resistome studies[25–28]. This is illustrated by the growth of the catalogue of known resistance genes, which has occurred alongside a rapid decrease in sequencing costs. A survey of resistance proteins in the UniProt database[29] demonstrates that since 1986 there has been an exponential increase in the number of hypothetical resistance determinants classified as β-lactamases, chloram phenicol acetyltransferases or tetracycline efflux pumps (FIG. 2a). The surfeit of sequencing data exemplifies the second era of resistance studies but in many cases, the lack of functional validation of these genes calls into question whether all of them are resistance determinants. Similarly, the sequence diversity that is captured using cutting-edge techniques may not translate into functional diversity. One technique that has proven useful in the discovery of resistance determinants that are novel, diverse and validated is functional metagenomics (BOX 3), which is a technique that has been developed to mine metagenomes for various phenotypes without a need for culturing[30]. Functional metagenomics has proven to be essential for the characterization of antibiotic resistomes. Briefly, this technique consists of cloning metagenomic DNA into expression vectors and screening for a

phenotype of interest (for example, antibiotic resistance) in a facile host organism. This bypasses the bottleneck that is imposed by bacteria that are difficult to culture and explicitly selects for genes that can spread by horizontal transfer, as only phenotypes that are transferable to a heterologous host are detected. However, antibiotic resistance that results from mutation of the antibiotic target, which is an important contributor to resistance in many organisms, such as *Mycobacterium tuberculosis*[31,32], may not always be detected in functional metagenomic screens, as a single wild-type copy of these genes in the expression host often confers a dominant susceptible phenotype. This is the case for quinolone resistance that results from mutations in the DNA gyrase enzyme[31], although the analysis of evolutionary patterns in such genes may enable antibiotic resistance risk estimates to be made[33–35]. Furthermore, the artificial action of extracting and cloning DNA can lead to the transfer of genes that otherwise might not be mobilizable, which possibly raises concerns in regard to the spread of resistance in cases in which the actual risk may be low, but it may also result in the underestimation of sequences related to mobility elements, which are not explicitly selected for in screens for antibiotic resistance.

The coupling of functional metagenomic selections with high-throughput sequencing and computational methods has facilitated an increase in our ability to catalogue resistomes from diverse habitats. Importantly, functional metagenomics can both ascribe a resistance function to known genes that were not previously identified as resistance determinants and discover resistance genes with novel sequences. For example, a recent analysis of functionally discovered resistance genes found that although many of the gene products had high amino acid identity to known enzymes, most had low identity to known resistance determinants[36] (FIG. 2b). Similarly, the utility of functional metagenomics in elucidating entirely new resistance functions is highlighted by its recent application to the discovery of novel rifamycin phosphorylases[37], dihydrofolate reductases[38] and tetracycline-inactivating enzymes[39].

The resistomes of different habitats

Functional metagenomics was first described in 2000 as a method for accessing the diverse biochemical activities encoded in the uncultured soil metagenome[30]. In an early application of functional metagenomics for the explicit purpose of screening for antibiotic resistance genes, a library that contained 5.4 Gb of metagenomic DNA from a remnant oak savannah site was selected against a panel of aminoglycoside antibiotics, as well as tetracycline and nalidixic acid[40]. Sequencing of 10 resistant clones revealed that all but one contained novel resistance determinants. Since these initial studies, functional metagenomics has been widely used to screen a diverse range of soil metagenomes, including remote arctic soils[41–43], apple orchard soils[44], alluvial soils[45,46], wetland soils[47], agricultural soils[38,48] and urban soils[49], and has identified novel genes that confer resistance to diverse classes of antibiotic. Agricultural resistomes are of particular interest because the selective pressures of heavy antibiotic use and extensive anthropogenic interaction make this a high-risk habitat for antibiotic resistance selection and dissemination. Indeed, the *mcr1* plasmid-mediated colistin resistance determinant was first discovered on a Chinese pig farm[50], although it has since been detected in new and banked clinical and environmental samples across the globe[51–59] (BOX 2).

In addition to soil habitats, human-associated resistomes have been a recent focus of functional metagenomic investigations. The first human-associated microbial community to be queried for antibiotic resistance by functional metagenomic selection was the oral microbiome. Screening of a small functional library constructed from dental plaque and saliva samples against tetracycline resulted in the discovery of *tet*(37), a gene that encodes a tetracycline-inactivating enzyme that has low sequence identity to any previously described tetracycline resistance determinant, as well as other resistance genes[60]. A larger follow-up study revealed resistance against tetracycline, gentamicin and amoxicillin in the oral microbiome[61]. The first report of a functional metagenomic interrogation of the human gut resistome screened metagenomic DNA from saliva and stool samples from two healthy humans against a panel of 13 antibiotics[62]. Sequencing of 95 unique inserts, which together conferred resistance to all of the 13 antibiotics that were screened, revealed that they were largely dissimilar to the closest known resistance genes. For example, 10 novel β-lactamases were identified in just two gut microbiomes. Remarkably, these microbiomes encompassed greater phylogenetic β-lactamase diversity than all previously described β-lactamases (FIG. 2c). Additional studies have examined the gut microbiomes of diverse human populations, including healthy adults[63], healthy twins and their mothers[18,64,65], preterm infants[36], uncontacted antibiotic-naive Amerindians[10], and families, as well as livestock and pets, from developing countries in Latin America[66]. Importantly, these studies have made it clear that, although the resident human-associated resistome is enriched after antibiotic therapy[36], the gut contains an extensive resistome even in the absence of anthropogenic antibiotic use[10,66].

Limitations of resistome studies

Although these studies are extremely valuable in cataloguing the genetic diversity of the global resistome, they have been largely descriptive or focused on collecting data, and thus require substantial downstream mechanistic validation. It is important to emphasize that just because a gene confers resistance when it is overexpressed in a heterologous host, it may not be a bona fide resistance gene in the original host. Indeed, nuanced definitions of antibiotic resistance should consider the function of resistance determinants in their native contexts and the risk of transmission of resistance determinants between bacterial species[35]. There is extensive evidence that resistome composition is strongly correlated with the phylogenetic structure of a microbial community, and that the synteny of antibiotic resistance genes with mobility elements is observed less frequently in environmental meta genomes (for example, those from soil) than in sequenced pathogen genomes[48,67] (FIG. 1b). Nonetheless, the discovery of identical resistance genes in multiple genomic and environmental contexts suggests historical transfer and/or dissemination events. For example, a recent functional metagenomic study identified an identical TEM-1 β-lactamase in 25 different habitats and genetic backgrounds[66] (FIG. 1a). This research, when viewed in light of recent emerging resistance threats (BOX 2), emphasizes that although not all resistance genes are threats for widespread dissemination, we should pay close attention to the ones that are. A focus of future studies on antibiotic resistomes should be the quantification of risk of horizontal gene transfer to pathogens based on 'metadata' associated with a given resistance gene (for example, synteny with mobility elements, whether it is plasmid-borne or chromosomal, and whether it is found in a close relative of a human pathogen) to model the risk of transmission[34,35].

Next-generation approaches to resistance

Strategies to combat antibiotic resistance will require substantial changes both to the antibiotic pipeline and stewardship practices, and to antibiotic resistance surveillance. The ubiquity of antibiotic resistance suggests that the drug development pipeline must include proactive screens for existing environmental resistance threats to new drugs and should explore alternative strategies for combating resistance outside of the traditional antibiotic pipeline. In addition, it is the responsibility of the wider medical and scientific communities to apply and combine the findings from the first two eras of resistome studies in efforts to surveil potential antibiotic resistance hotspots for emerging resistance threats before they spread.

Next-generation therapeutics

A key aspect that requires investigation in the next generation of antibiotic resistance studies is the proactive development of therapeutic strategies to counteract emerging antibiotic resistance. One side of this approach involves having a greater focus on resistance potential when developing new and improved antibiotics. Current antibiotic discovery and development pipelines only take into account resistance mechanisms that are already prevalent in the clinic. In retrospect, we know that this strategy is doomed to fail, as resistance has been observed for every antibiotic that has been implemented for human use[17]. Going forwards, we should improve on the current reactive strategies by screening promising lead compounds for resistance against diverse functional metagenomic libraries. This will identify existing mechanisms of resistance that are likely to disseminate to the clinical setting following the introduction of the antibiotic for clinical use (FIG. 3).

In the near future, our understanding of the resistome will be sufficiently mature to enable the development of strategies that actively combat resistance mechanisms themselves in contrast to current strategies that side-step resistance through the expensive development of new antibiotics that immediately select for new mechanisms of resistance. This new approach, in our opinion, provides a higher likelihood of success than searching for a fabled 'silver bullet' antibiotic to which resistance is unlikely to develop. One such strategy is the repurposing of existing antibiotics in synergistic combinations. For example, consider the triple β-lactam combination of meropenem, piperacillin and tazobactam, which synergistically kills methicillin-resistant *S. aureus* (MRSA) N315 *in vitro* and in a mouse infection model[68]. The bacteria were `collaterally sensitive` to the combination of these three antibiotics, and thus the combination suppressed the evolution of further resistance[68]. Treatment regimens that induce collateral sensitivity not only enable the elimination of resistant bacteria but also subject resistant bacteria to selective pressure to discard their resistance gene[69]. Inverting the selective advantage of antibiotic resistance is a promising approach for slowing the rate at which resistance evolves in a population. A recent highlight in the search for selection-inverting compounds involved a screen of nearly 20,000 small molecules for compounds that select against the tetracycline efflux pump TetA. This screen identified two candidates that successfully selected for the loss of the *tetA* gene[70]. Perhaps more important are drugs under development and in early-phase testing that target AcrA–AcrB–TolC efflux complexes in Gram-negative bacteria. Unlike TetA proteins,

which are tetra cycline-specific, AcrA–AcrB–TolC pumps have broad substrate tolerance and can confer multidrug resistance, particularly in Gram-negative bacteria[71].

A final strategy that has already proven to be effective in opposing widespread resistance is the inhibition of antibiotic resistance enzymes[72]. Small molecules that lack antibiotic activity but enhance the efficacy of antibiotic therapy are broadly termed antibiotic adjuvants[73]. β-Lactamase inhibitors and TetA efflux selection inversion[70,72,74] fall in this category, as do promising examples from recent studies that paired antibiotics with repurposed drugs that are already on the market for other indications, such as the opioid-receptor agonist loperamide, which facilitates tetracycline uptake, and the ADP receptor antagonist ticlopidine, which inhibits teichoic acid biosynthesis[3,73,75,76]. The continued discovery of antibiotic adjuvants is facilitated and informed by resistome studies that comprehensively describe the collection of possible resistance genes. For example, consider the recently described *E. coli*-hosted array of more than 40 mechanistically distinct and functionally validated resistance genes[77]. This platform, which represented resistance to 16 classes of antibiotics, was used to screen a limited small-molecule library for compounds that could enhance the antibiotic activity of gentamicin. Of the 27 compounds that exhibited this activity, eight were found to inhibit aminoglycoside-$2''$-O-nucleotidyltransferase (ANT($2''$)-Ia)-mediated resistance. Two compounds that were selected for further validation exhibited poor antibiotic activity alone and were confirmed to inhibit ANT($2''$)-Ia *in vitro*. By screening drug combinations against a phenotypic resistome array, the authors were able to identify inhibitors of antibiotic resistance genes, thus demonstrating the utility of leveraging resistome data to discover adjuvants that can prolong the efficacy of existing antibiotics[77].

Next-generation surveillance

Lastly, it is important to prioritize continued and increased surveillance of the antibiotic resistome, particularly hotspots in which there is a high likelihood of resistance gene evolution and transfer between organisms (FIG. 3). These hotspots include, but are not limited to, agricultural settings in which large quantities of antibiotics and human pathogens, as well as environmental microorganisms, coexist and potentially exchange DNA[50]; hospitals in which selective pressure is omnipresent owing to the extensive use of antibiotics and antimicrobials, which may co-select for antibiotic resistance[24]; and wastewater and sewage systems, which directly expose the human gut microbiota to many pharmaceutical residues[66]. Surveillance requires regular testing of pathogens, commensals and microbial communities for resistance to a broad range of antibiotics. This can occur using culture-dependent or genomic approaches, and necessarily entails the continued development of technologies that will facilitate the identification of antibiotic resistance determinants.

Recent computational and bioinformatic advances have substantially improved our ability to identify antibiotic resistance genes from PCR amplicons and whole-genome sequencing (WGS)[27]. These databases contain nucleotide or protein sequences for known antibiotic resistance determinants, and can be queried (by BLAST[78], for example) to identify the antibiotic resistance genes that are present in a given genome or metagenome. However, these functional predictions are explicitly based only on sequence data and are vulnerable to

false-positive predictions, particularly when functional assignments are based on annotations that have not themselves been directly validated biochemically or phenotypically. A key component of our ability to annotate and predict antibiotic resistance genes from genomic data is the continued improvement of antibiotic resistance gene databases through functional validation[79–84]. In addition, improved probabilistic annotation algorithms, such as profile hidden Markov models, have been shown to perform substantially better than pairwise sequence alignment in terms of precision and recall[85,86].

For example, Resfams, a curated database of 166 profile hidden Markov models that were constructed from functionally validated families of antibiotic resistance proteins, correctly predicted 45% more β-lactamases in functionally selected metagenomic contigs than BLAST alignment to antibiotic resistance databases[84]. In other studies, nucleotide sequences from metagenomic datasets were used to computationally predict *qnr* genes that might confer resistance to quinolone[87,88]. Although *qnr* genes have low similarity to other resistance determinants and relatively high in-family diversity, the authors were able to discover and functionally verify several novel variants[87,88]. As a result of the development of these tools, we are now able to more confidently identify both transferable and intrinsic resistance determinants from genomes and metagenomes. Going forwards, it is important that these databases are regularly updated with the nucleotide and amino acid sequences of functionally validated, emerging resistance determinants (FIG. 3).

Despite these advances, the prediction of resistance phenotype on the basis of genotype remains a challenge. WGS-based methods are a promising approach to improve the speed and accuracy of diagnostics[89–91]. An additional advantage of WGS-based diagnostics is the possibility for the improved identification of antibiotic resistance by target modification, which is often not feasible using existing PCR-based or microarray-based methods[91–93]. A current highlight of sequencing-based diagnostics is the Mykrobe predictor, which identified antibiotic susceptibility profiles for 12 antibiotics from raw, unassembled Illumina sequencing data from *S. aureus* and *M. tuberculosis* isolates, as well as from *in silico*-simulated heterogeneous populations, with high sensitivity and specificity[94]. A recently described alternative approach uses draft genome assemblies as inputs and a machine-learning approach to accurately predict resistance profiles for multidrug-resistant Enterobacteriaceae isolates[86]. Although it is currently cost-prohibitive and time-prohibitive to use WGS as a clinical diagnostic tool, continued reductions in sequencing costs and improvements in computational algorithms may soon make this approach feasible. Faster and more accurate diagnostics will considerably decrease the number of empirical and unsuitable antibiotic prescriptions, thus limiting further selection for resistance (FIG. 3).

Two recent examples from the literature highlight the importance of surveillance efforts as we attempt to prevent the spread of antibiotic resistance. These examples pertain to the current last-resort antibiotics against carbapenem-resistant members of the Enterobacteriaceae: namely, colistin and tigecycline. The first example is the emergence of the *mcr1* gene as a transmissible threat to colistin use. The seminal example of a plasmid-borne colistin-resistance gene was found through an active surveillance programme in China that monitored antimicrobial resistance in animals that were reared to be consumed by humans[50]. Soon after its discovery, several retrospective studies demonstrated that plasmid-

borne *mcr1* had already spread worldwide and into the clinic[95] (BOX 2). In this case, surveillance provided crucial early detection. The second example of the importance of surveillance relates to tigecycline, a `semi-synthetic` tetracycline antibiotic[96]. Owing to its increased affinity for the bacterial ribosome compared with other tetracyclines, and to the presence of a glycyl tail, tigecycline is subject to reduced resistance through ribosomal protection or drug efflux[97]. However, tigecycline is sensitive to the tetracycline-inactivating protein TetX[98]. Although this protein was originally discovered in *Bacteroides fragilis* — a usually non-pathogenic human gut commensal — several clinical isolates have recently tested positive for *tetX*[99,100], and tetracycline inactivation has been discovered to be more widespread in environmental metagenomes than was previously thought[39]. Continued surveillance of this apparent emerging resistance mechanism is prudent, as tigecycline and other next-generation tetracycline derivatives are being designed to exclusively evade resistance through ribosomal protection and efflux[101,102]. The increased use of next-generation tetracyclines imposes a strong selective pressure on the acquisition and spread of tetracycline-inactivating enzymes from the environmental resistome. Foresight of this event should enable the proactive development of next-generation therapeutic strategies before the widespread dissemination of a resistance threat.

Conclusions

Extensive study of the antibiotic resistome throughout the past few decades has enabled us to begin to understand and address existing antibiotic resistance threats. However, it is evident that antibiotic resistance will continue to evolve and spread despite our best efforts to develop new antibacterial agents. Unless current practice is changed, it will be increasingly difficult to establish and maintain even a transient lead over bacteria in this 'arms race'. As research into antibiotic resistance expands, it is important to adopt an explicitly proactive approach to antibiotic resistance identification and surveillance, as well as antibiotic therapy development. This proactive approach involves using a combination of functional metagenomics, `next-generation sequencing` and cutting-edge computational methods to monitor the evolution and dissemination of resistance before a given resistance determinant emerges in a pathogen or in the clinical setting, as well as proactively developing next-generation therapies that target these resistance determinants. The spread of plasmid-borne carbapenem, quinolone and polymyxin resistance in recent years is a sobering reminder of our need to mitigate existing threats and to anticipate emerging resistance mechanisms before they circulate widely in the clinical setting if we wish to alter the current bleak antibiotic resistance trajectory. Recent advances in the field highlight the promise that the next generation of resistome studies hold for characterizing and countering emerging resistance threats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Glossary

Antibiotics

Drugs that inhibit the growth of bacteria or kill them

Natural products

Small molecules that are naturally produced by living organisms

Proto-resistance genes

Genes that have the potential to evolve a resistance function

Cryptic resistance genes

A resistance gene that is embedded in a bacterial chromosome, but that is not obviously associated with antibiotic resistance. The respective gene is usually either not expressed or expressed at low levels

Metagenomes

The collective genetic material in a given environment

Synteny

The occurrence of multiple genes in the same genetic locus

Horizontal gene transfer

The transmission of genetic material between bacterial organisms by transformation, transduction or conjugation, in contrast to vertical transmission through heredity

Axenic

A term that describes a culture that contains only a single species

Koch's postulates

A series of criteria proposed by Robert Koch in 1890 that can be used to establish a causal relationship between a microorganism and a disease

Collaterally sensitive

A term that describes organisms that develop resistance to one antibiotic and, as a result of the new mutation, are more sensitive to another antibiotic

PCR

A molecular biology technique that is used to amplify nucleic acids of known sequence

Whole-genome sequencing

(WGS). The use of next-generation sequencing to determine the complete sequence of an organism's genome

Hidden Markov models

Statistical models that are widely used in biological sequence analysis and annotation

Semi-synthetic

A term that describes a small molecule that is produced by chemical modification of a natural product

Next-generation sequencing

High-throughput nucleic acid sequencing technologies that have emerged in the past few decades to enable substantial increases in sequencing capacity

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Box 1

Common mechanisms of antibiotic action and antibiotic resistance

Antibiotic targets are generally conserved across the bacterial domain of life, and are absent from or sufficiently different in eukaryotes. Although hundreds of such targets exist in theory, our current antibiotic arsenal generally attacks the bacterial ribosome (inhibiting protein synthesis; for example, aminoglycosides); cell wall synthesis and lipid membrane integrity (compromising membrane integrity or lysing bacterial cells; for example, β-lactams); single-carbon metabolic pathways (inducing metabolic disruption; for example, sulfonamides); and DNA maintenance (interfering with bacterial replication and genomic integrity; for example, quinolones)[2,17,103] (see the figure, top).

Bacteria typically resort to one of a few common strategies to resist antibiotics[104] (see the figure, bottom). One resistance mechanism involves the inactivation of the antibiotic through enzymatic degradation or modification of the antibiotic scaffold, which renders it ineffective. Examples of this mechanism include chloramphenicol acetyltransferases[105], tetracycline-inactivating enzymes such as TetX[39,106] and, most notably, the widespread β-lactamases[107,108]. A second strategy for resistance is protection, alteration or overexpression of the antibiotic target. This approach is used by vancomycin-resistant enterococci, which enzymatically modify peptidoglycan, thus decreasing the affinity of vancomycin for its target[109], and by methicillin-resistant *Staphylococcus aureus* (MRSA), which expresses a redundant methicillin-insensitive variant of the native penicillin-binding protein[110]. Two additional resistance mechanisms rely on keeping the antibiotic out of the bacterial cell, through either efflux or altering the permeability of the cell membrane. By expressing either a generalist multidrug efflux pump[111–114] or an antibiotic-specific exporter, such as tetracycline efflux pumps[115], bacteria keep the intracellular concentration of the antibiotic below inhibitory levels. Alternatively, some bacteria reduce their cell membrane or cell wall permeability to prevent antibiotics from entering the cell by decreasing porin expression or expressing a more selective porin variant[116,117]. In some cases, bacteria will use several complementary mechanisms to achieve high levels of resistance. For example, high-level carbapenem resistance in clinical isolates of *Enterobacter cloacae* that lack any known carbapenemase can be achieved through a combination of porin mutations that decrease carbapenem uptake and increased expression of a chromosomal non-carbapenemase β-lactamase[118].
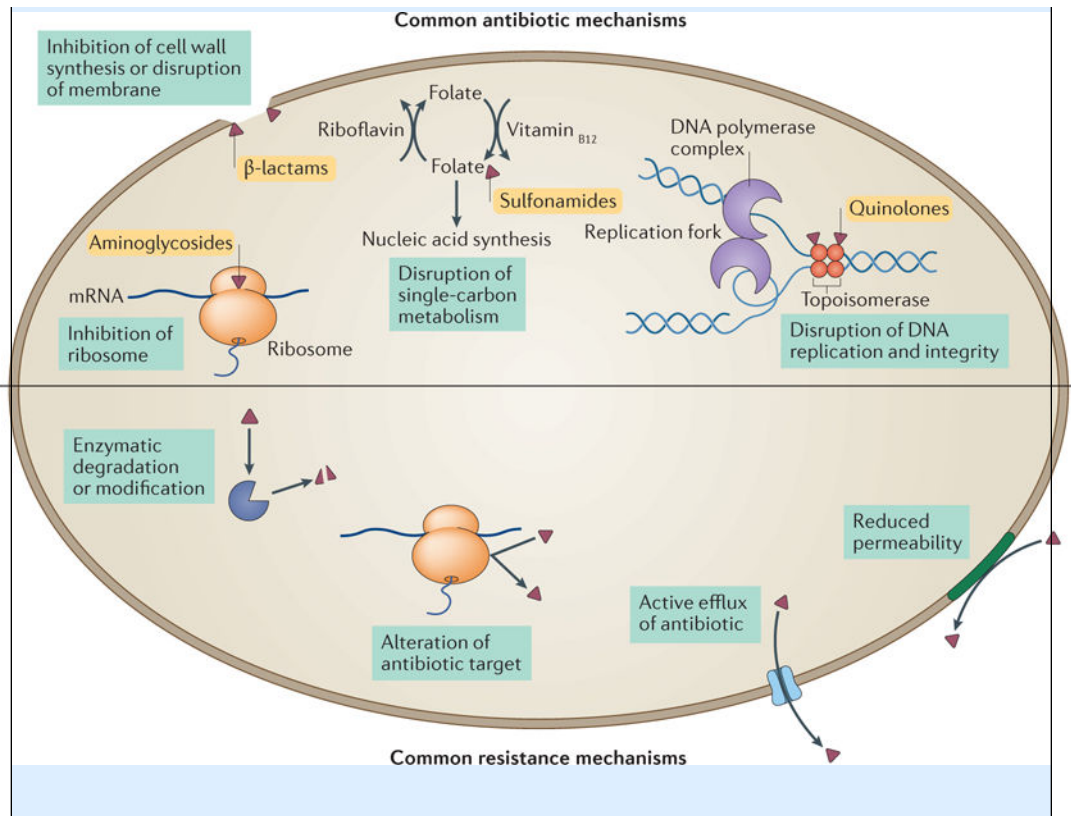
Box 2

Global spread of plasmid-borne resistance

In the past several decades, various new antibiotic resistance threats have emerged and spread worldwide. In particular, the emergence and spread of plasmid-borne resistance genes (see the figure and Supplementary information S1 (table) for specific reports of plasmid-borne resistance) — which are likely to undergo horizontal gene transfer — have jeopardized many last-line antibiotic therapies, including the cephalosporin and carbapenem families of β-lactam antibiotics, quinolones, such as ciprofloxacin, and an antibiotic of last resort, colistin[5]. Cephalosporins have been compromised by resistance owing to extended spectrum β-lactamases (ESBLs), which were first reported in 1979 (REF.[119]), and subsequently spread phylogenetically across the Enterobacteriaceae and geographically across the globe[120]. Consequently, many infections that were previously treated by drugs such as cefotaxime must now be treated with the carbapenem class of antibiotics. However, the spread of carbapenemases[121] — particularly New Delhi metallo-β-lactamase 1 (NDM1)[122–124] and *Klebsiella pneumoniae* carbapenemases (KPCs)[125] since their respective discoveries in 2006 and 2001 — has also rendered the carbapenem class of antibiotics vulnerable[126] (see the figure).

A similar development has been observed for the quinolones, which were originally touted to be at low risk of displaying the type of transferable resistance that has beleaguered the β-lactam antibiotics. Indeed, whereas chromosomal resistance to this class of antibiotic can readily develop through mutations in DNA gyrase, transferable resistance was slow to develop following the clinical introduction of these antibiotics in the 1960s. Nonetheless, plasmid-mediated resistance through the quinolone resistance (QNR) proteins, first described in 1998, has emerged from quinolone overuse and has subsequently spread worldwide (see the figure). In the search for the origins of QNR resistance determinants, a PCR screen of Gram-negative organisms found homologues in the aquatic bacterium *Shewanella algae* that have almost 99% amino acid identity[127]. *Shewanella* species that lack these homologues were found to have a fourfold to eightfold greater sensitivity to quinolone antibiotics than those that expressed these homologues, which indicates the ability of the *qnr*-related genes to confer some antibiotic resistance even in their native contexts, in which they probably bind to DNA for some other purpose[2,127]. The hypothesized horizontal transfer of *qnr* homologues from environmental to pathogenic bacteria corroborates hypotheses that propose that many resistance genes may have been co-opted by pathogens from the environmental resistome[13,14,127,128]. Quinolone efficacy has been further reduced by the emergence of the plasmid-borne bifunctional aminoglycoside and quinolone resistance gene *aac(6′)-Ib-cr* in Shanghai in 2003 (REF.[129]). This gene, which differs from that encoding a classical aminoglycoside transferase by only two base pairs, is notable in its ability to modify fluoroquinolones and aminoglycosides through acetylation. Alarmingly, *aac(6′)-Ib-cr* is often colocalized with other resistance genes, such as ESBLs[130], on multidrug resistance plasmids.

The spread of the aforementioned resistance mechanisms leaves only a few classes of antibiotic approved for the treatment of infection by carbapenem-resistant members of

the Enterobacteriaceae. One of these, the polymyxin class of peptide antibiotics, was first used in the 1960s but was abandoned in favour of other antibiotics owing to its toxicity. Recently, the use of the polymyxin colistin has been revived owing to its efficacy against several multidrug-resistant Gram-negative pathogens[131]. However, transferable resistance has now begun to threaten colistin. In 2015, a colistin-resistant *Escherichia coli* isolate from a pig farm in Shanghai was found to contain *mcr1* — a plasmid-borne gene that encodes a phosphoethanolamine transferase[50]. This finding, and the subsequent discovery of *mcr1* on plasmids worldwide[51–59] (see the figure), foreshadows the fall of colistin as a last-resort antibiotic that lacks transferable plasmid-encoded resistance. With the high levels of antibiotic prescription in medicine and unregulated antibiotic use in agriculture around the world it is likely that it is only a matter of time before completely pan-resistant plasmids are found that combine the above resistance mechanisms. Indeed, the coexistence of NDM1 and MCR1 in members of the Enterobacteriaceae has already been observed[55,132], and a recent report documented the co-transfer of *mcr1* and *ndm5* on a mobile hybrid IncX3–X4 plasmid[133].
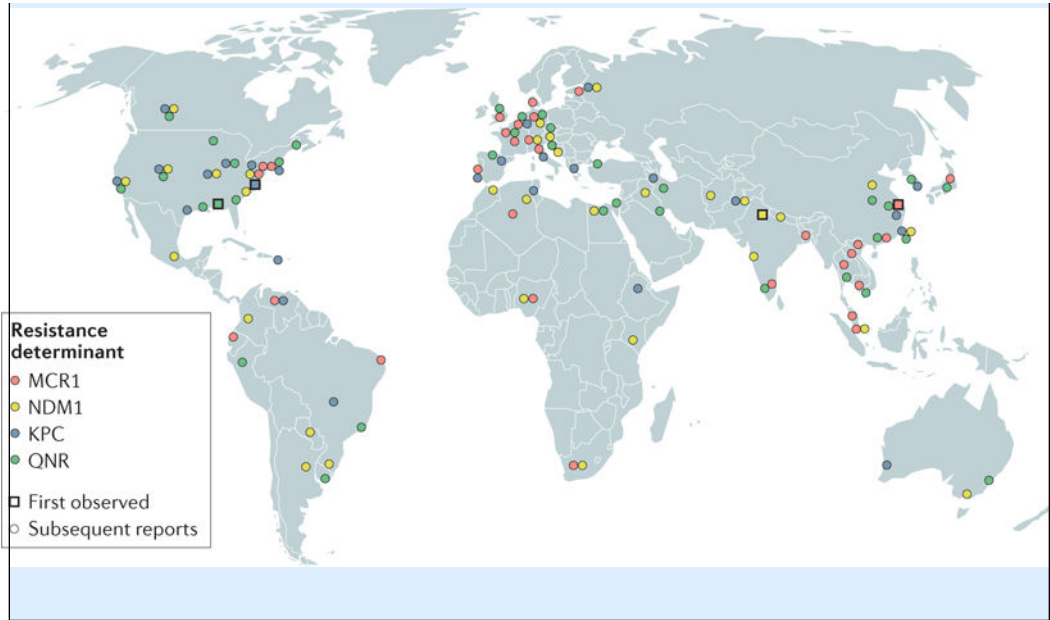
Box 3

Functional metagenomic approaches for characterizing resistomes

Functional metagenomics is a powerful culture-unbiased and sequence-unbiased tool[134]. A metagenomic library is created by extracting total metagenomic DNA from a microbial community, shearing the DNA to a target size distribution and cloning these fragments *en masse* into a screening vector (see the figure). The metagenomic library is then transformed into a heterologous host and selected for a phenotype of interest by, for example, culturing with antibiotics at a concentration that is lethal to the wild-type host[27,135]. Pairing functional metagenomic selection with next-generation sequencing and better sequence assembly and annotation algorithms[14] has simultaneously decreased the cost and increased the throughput of functional metagenomic experiments, and this has facilitated the characterization of resistomes from diverse habitats[10,18,36,48,66]. Importantly, there is no need to culture members of the target microbial community, and novel resistance genes can be discovered. Furthermore, the genes discovered by functional metagenomics are, by definition, candidates for horizontal transfer, as they must be functional in a heterologous host.

However, there are several limitations of functional metagenomic approaches. For a gene to be identified in a functional metagenomic screen, it must be functional in a heterologous host. Common hosts, such as *Escherichia coli*, probably present a poor system for the discovery of resistance genes that originated from Gram-positive organisms. Indeed, poor overlap has been reported between clones recovered from the same functional metagenomic library that were subjected to the same screen but in different hosts[136,137]. In addition, it is generally difficult to screen Gram-positive bacteria-specific antibiotics in *E. coli* or other common Gram-negative platforms owing to their innate resistance, although in some cases this may be overcome[138]. Thus, there is a clear need to develop and screen functional libraries in phylogenetically diverse heterologous hosts[134,139]. Furthermore, functional metagenomic experiments may oversample resistomes, as the method struggles to distinguish resistance genes that pose clinical threats from those that are unlikely to spread beyond their current environment. In addition, genes that confer antibiotic resistance when overexpressed in a heterologous host may not be genuine resistance genes in their native contexts[35]. To reduce the risk of false-positives, it is important that novel resistance genes are validated using complementary microbiological and biochemical methods, and are cross-checked against existing databases. Finally, the removal of an antibiotic resistance gene from its genomic context often also removes positional information that is pertinent to assessing its risk of dissemination. This can be overcome, in part, by altering insert size, as larger inserts may contain informative regions that flank resistance genes, including mobility elements or plasmid sequences that might indicate the risk of spreading (that is, the mobilome)[48,66,140].
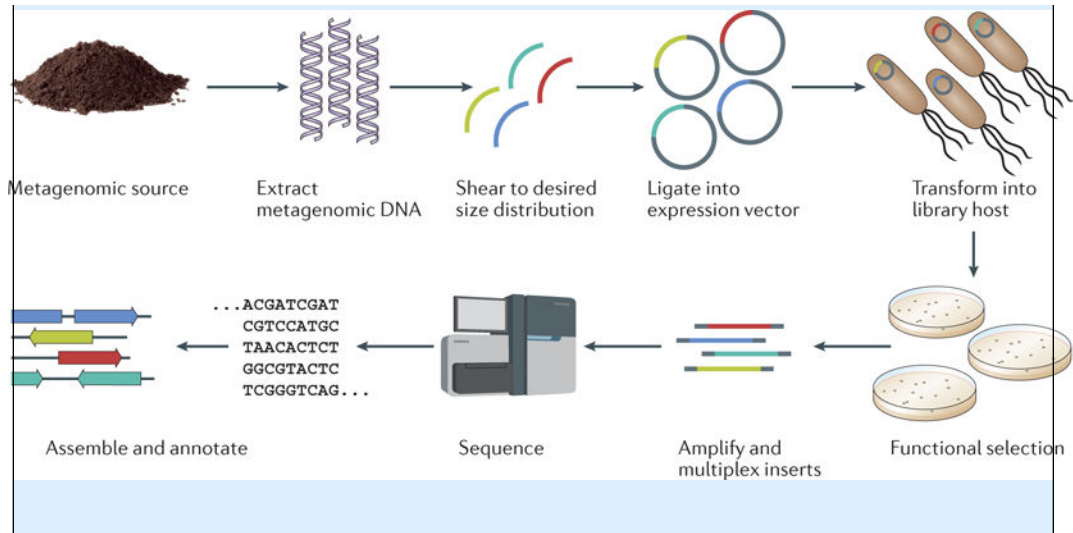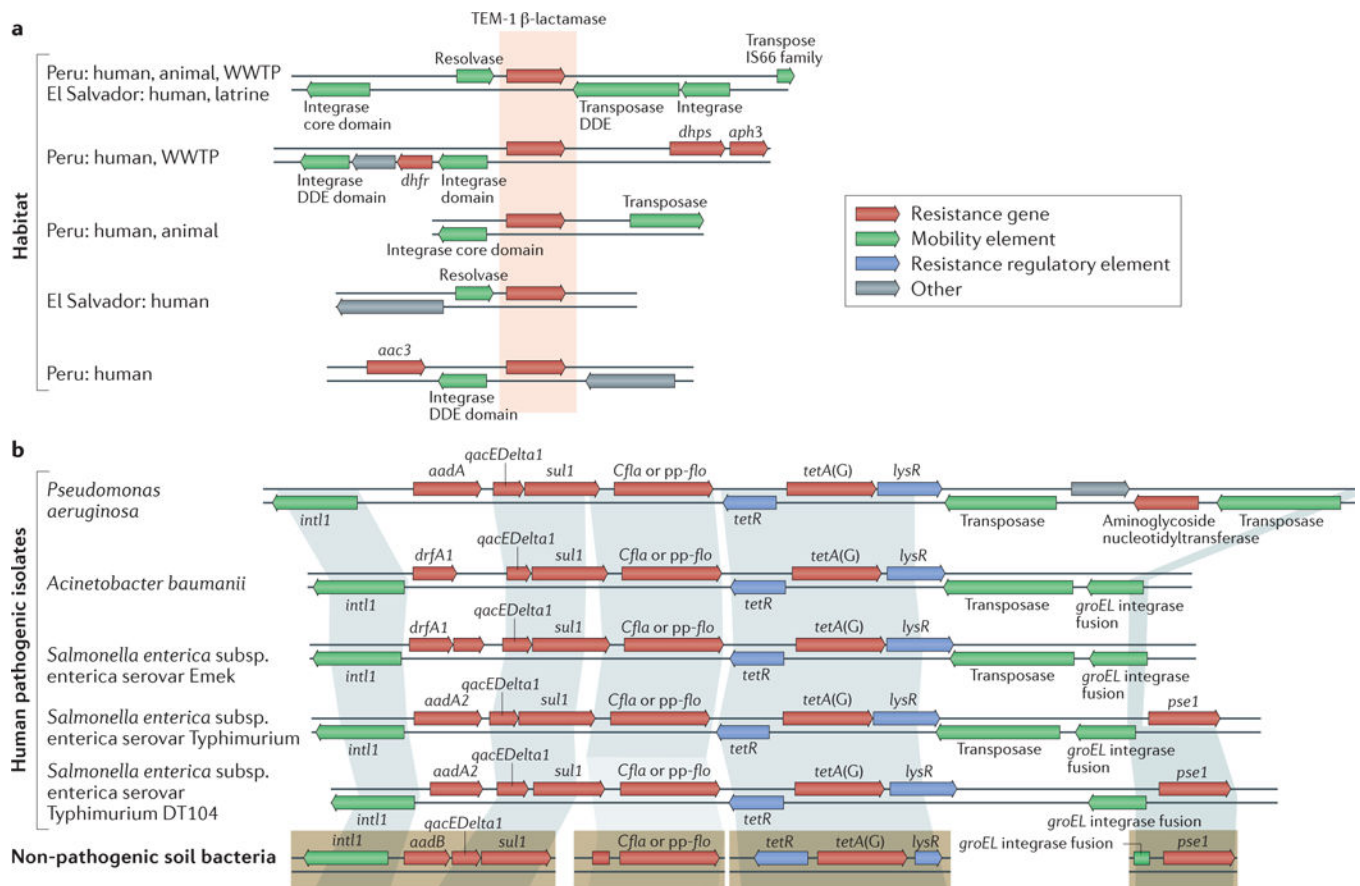
**Figure 1. The synteny of antibiotic resistance genes provides historical context and foreshadows future**

The genetic context of antibiotic resistance genes can provide important evidence regarding the likelihood of past or future horizontal gene transfer. **a** | A single β-lactamase (TEM-1) was found in 25 different genetic contexts in a recent cross-habitat resistome study, which provided a historical record of past mobility. Five contexts are shown here. Habitats in which a gene was discovered are indicated on the left ('human': human-associated microbial community; 'animal': animal-associated microbial community; 'WWTP': wastewater treatment plant; 'latrine': composting latrine). **b** | Four resistance determinants that were discovered in functional selections of soil metagenomic libraries (bottom) have high identity with resistance genes from clinical pathogenic isolates. Shading indicates >99% nucleotide identity, which suggests recent horizontal transfer. Resistance genes in pathogens are syntenic with mobility elements and other resistance genes, which suggests that they may be present on a mobile multidrug resistance cassette and are therefore likely to undergo horizontal gene transfer. DDE, Asp-Asp-Glu. Part **a** is adapted with permission from REF.[66], Macmillan Publishers Limited. Part **b** is adapted with permission from REF.[14], AAAS.
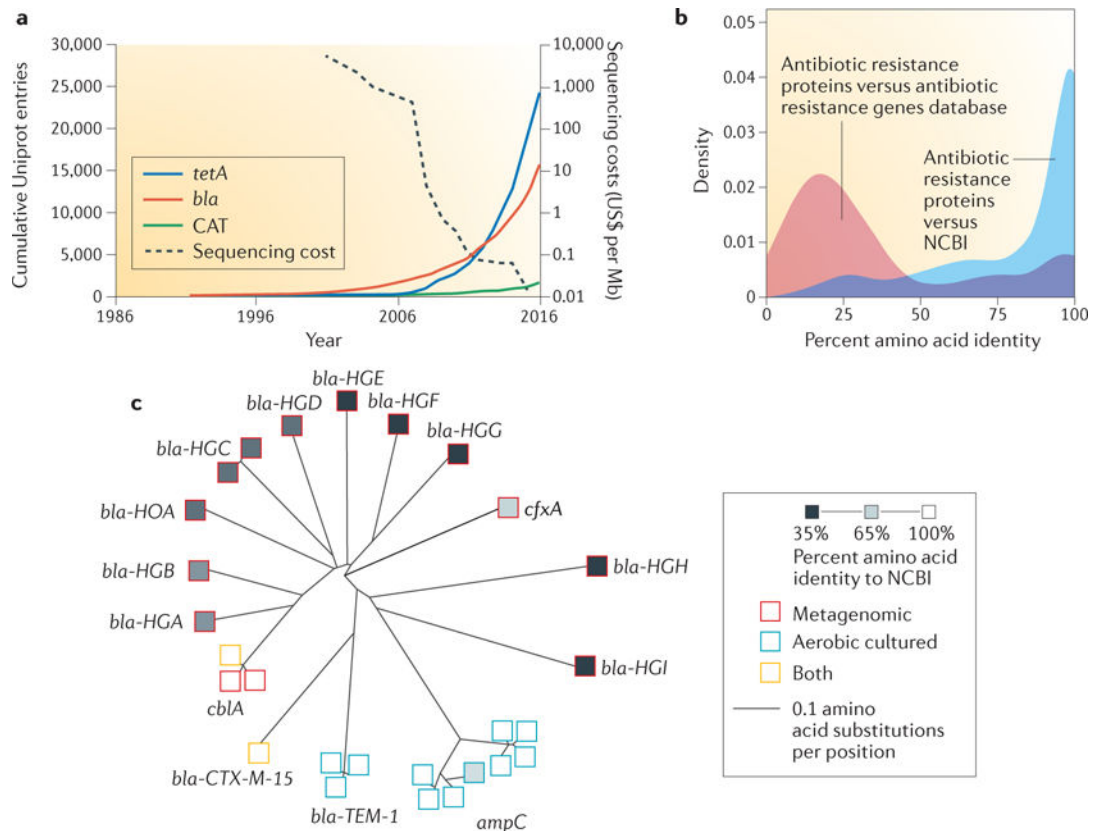
Figure 2. Next-generation sequencing and functional metagenomic selection accelerate the cataloguing of known and novel resistance genes

a | The catalogue of resistance genes has increased exponentially in the second era of antibiotic resistome studies owing to improvements in next-generation sequencing. This trend is exemplified by the tetracycline efflux pump-encoding gene *tetA*, by the *bla* family encoding β-lactamases and by chloramphenicol acetyl transferase (CAT). Data from the UniProt database[29], which was accessed on 12 October 2016; the database was searched using Enzyme Commission numbers (*bla*: 3.5.2.6; CAT: 2.3.1.28) or a family name (*tetA*). Data on sequencing costs are from National Human Genome Research Institute webpage[28]. **b** | Functional metagenomics is a powerful tool for ascribing antibiotic resistance function to known gene products. Antibiotic resistance proteins identified by functional metagenomic selection of the preterm infant gut microbiome have high amino acid identity to proteins in the National Center for Biotechnology Information (NCBI) database but low amino acid identity to proteins in antibiotic resistance databases. **c** | In addition, functional metagenomics can reveal entirely sequence-novel resistance determinants. β-Lactamases that were identified by functional metagenomic selection of two human gut resistomes (red squares) encompassed greater phylogenetic diversity than all previously identified classes of β-lactamase (blue squares). Part **b** is adapted with permission from REF.[36], Macmillan Publishers Limited. Part **c** is adapted with permission from REF.[62], AAAS.
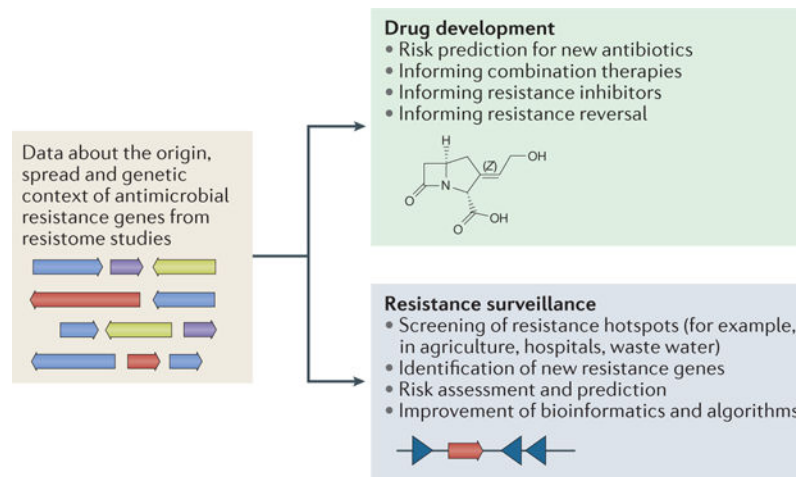
Figure 3. The integration of next-generation sequencing and screening technologies with drug development and resistance surveillance

The wide application of functional metagenomic selections and next-generation sequencing will steadily increase our knowledge of functionally annotated antibiotic resistance genes and their genetic context. This will enable more intelligent antibiotic resistance surveillance and drug design by accounting for specific mechanisms of resistance and the risk of resistance spread.