

The antibiotic resistome: the nexus of chemical and genetic diversity

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Abstract | Over the millennia, microorganisms have evolved evasion strategies to overcome a myriad of chemical and environmental challenges, including antimicrobial drugs. Even before the first clinical use of antibiotics more than 60 years ago, resistant organisms had been isolated. Moreover, the potential problem of the widespread distribution of antibiotic resistant bacteria was recognized by scientists and healthcare specialists from the initial use of these drugs. Why is resistance inevitable and where does it come from? Understanding the molecular diversity that underlies resistance will inform our use of these drugs and guide efforts to develop new efficacious antibiotics.

Antibiotics are arguably the most successful form of chemotherapy developed in the twentieth century and perhaps over the entire history of medicine. The first systematic searches for antimicrobial agents were carried out at the end of the nineteenth century in the wake of the acceptance of the 'germ theory of disease', which followed the conclusive experiments of researchers including Pasteur and Koch. Ehrlich's pioneering small molecule screening approaches and the successful identification of the first anti-trypanosomal and anti-syphilitic 'magic bullet' drugs ushered in the modern era of antimicrobial therapy¹. The first well-characterized antibacterial agents were derived from synthetic efforts, and emerged from the new science of synthetic organic chemistry that sustained the European dye industry.

However, it was the discovery of exquisitely potent, chemically diverse, and relatively non-toxic antibiotics derived from environmental bacteria and fungi, exemplified by the penicillin family, that shifted the antimicrobial drug-discovery paradigm from synthesizing small molecules to exploiting natural products. These discoveries paved the way for the 'golden era' of antibiotic discovery (1945–1960) during which most of the chemical classes of antibiotics now in clinical use were first characterized (see **TIMELINE**). This period was followed by the extensive medicinal chemical elaboration of these chemical scaffolds over the next decade (1970–1980) to tailor these drugs to improve pharmacology and evade antibiotic resistance — the 'golden age of antibiotic medicinal chemistry'. Relatively few antibiotic scaffolds are purely synthetic in origin. The lipopeptide daptomycin and the oxazolidinone linezolid were approved by

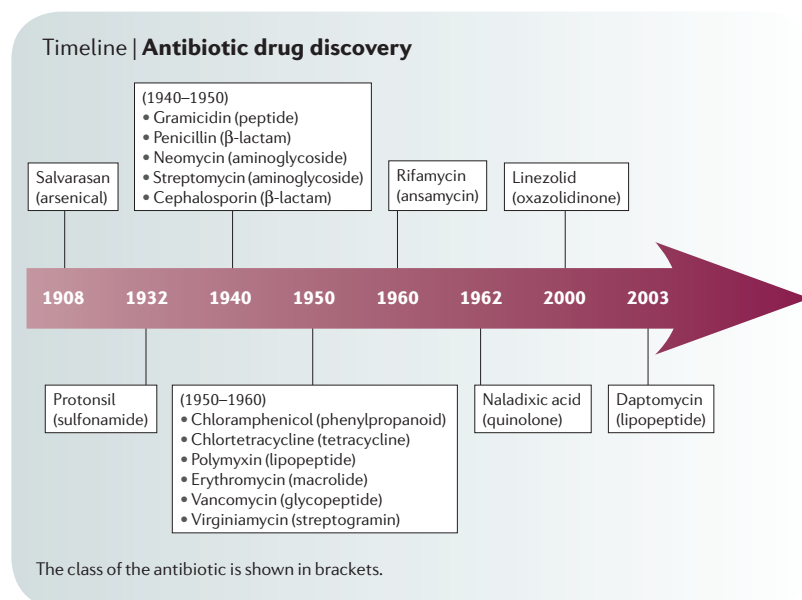
the FDA in 2003 and 2000, respectively, but daptomycin and the oxazolidinone class were both first discovered in the mid 1980s.

The pace of the discovery of new and clinically useful classes of antibiotics from the early 1960s to the present day has dramatically slowed. The reasons for this are complex and include the challenge of identifying new classes of antimicrobial agents from the natural product or synthetic small molecule collections available to the antibiotics industry, and a shift in focus after ~1960 in antibiotic drug development from discovery to medicinal chemical modification of existing antibiotic chemical scaffolds. Economic reasons for the decrease in discovery of new antibiotics include the number of many older off-patent agents that negatively influence drug pricing (antibiotics are among the cheapest drugs), the poor results of target-based drug discovery over the past decade, pressure to use new compounds sparingly to avoid resistance, and the perception that increasingly stringent criteria of regulatory agencies for new drugs are a barrier to bringing products to the market^{2–4}.

The problem of antibiotic resistance

One of the most important and sustained driving forces for antibiotic discovery over the past half century has been antibiotic resistance. Simply put, resistance is the continued growth of microorganisms in the presence of cytotoxic concentrations of antibiotics. Resistance is therefore relative, and as a result is defined operationally. In infectious disease clinical practice, antibiotic resistance that results in clinical failure is governed by the bioavailability of the antibiotics.

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Although resistance has been a continuing problem since antibiotics were introduced, it is the increase in the number, diversity and range of resistant organisms that has become a huge clinical problem⁵. Some 50 years after the first clinical use of penicillin, which was the harbinger of a new era in infectious disease medicine, some infectious organisms such as multidrug-resistant *Acinetobacter baumannii*⁶ and *Klebsiella pneumoniae*⁷ are now virtually untreatable with currently available antibiotics.

The multiply antibiotic resistant bacteria — the so-called superbugs — are now one of the most challenging problems faced by modern medicine^{8,9}. There are at least two classes of superbugs. The first class are well-known pathogens, many of which are classified in the same genera and species as the normal human commensal flora, but which have acquired antibiotic resistance genes and frequently have increased virulence¹⁰. Former commensal organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and drug-resistant *Escherichia coli* fall into this class. The second class is opportunistic pathogens^{6,11}, which are frequently environmental in origin and generally only infect very sick or immunocompromised patients. Opportunistic pathogens include *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *A. baumannii* and *Burkholderia cepacia*. The bacterial opportunists are frequently intrinsically resistant to multiple antibiotics.

Whether of commensal or environmental origin, these resistant bacteria might have modified drug targets, reduced the influx of antibiotics, exported antibiotics through the expression of efflux pumps, or inactivated antibiotics through the expression of specific enzymes to generate a 'perfect storm' of resistance features that renders superbugs untreatable with currently available antibiotics.

Superbug

A bacterial pathogen that is resistant to multiple antibiotics.

As medical practice improves, the population of immunologically vulnerable patients — the very young, the very old, individuals who are taking immunosuppressive drugs or those who have immunosuppressive diseases — is increasing. Unfortunately, the frequency of infection with opportunistic pathogens has also increased. This has resulted in an increased frequency of hospitalization, and hospitalization for longer periods, with increased exposure to multidrug-resistant pathogens that are present in healthcare settings. In the past, most pathogens resistant to multiple antibiotics were isolated from healthcare settings, in which antibiotic use was prevalent. Now, however, MRSA is routinely isolated from the community and, worryingly, antibiotic resistance is associated with increased virulence¹². Penicillin-resistant *Streptococcus pneumoniae* (PRSP), a common pathogen of children, is also mainly community-acquired¹³.

These factors threaten the future continued clinical use of antibiotics. Searching for new antibiotics or modifying existing chemical scaffolds will always be at best a temporary fix. What is it about antibiotic resistance, its evolution and origins that makes antibiotic resistance inevitable? Sequencing microbial genomes, cataloguing known and emerging resistance in the environment and clinics, and biochemical approaches have begun to uncover the molecular origins of antibiotic resistance.

Molecular mechanisms of antibiotic resistance

An integrated network of antibiotic resistance elements in bacteria provides protection against chemical threats. In bacteria, the front line of this resistance system is the cell envelope. In Gram-negative bacteria this includes the outer membrane, which is composed of an asymmetric lipopolysaccharide-phospholipid bilayer, and provides an effective physical barrier to the entry of molecules (including many antibiotics) into the cell. A cadre of outer-membrane spanning porins that facilitate the entry of small molecules into cells also passively excludes many antibiotics. In Gram-positive bacteria the absence of an outer membrane results in increased sensitivity to many antibiotics. Nonetheless, many Gram-positive bacteria, such as *Mycobacteria* species¹⁴, can thwart the cytotoxic effects of antibiotics through physiological defences. All bacterial genomes include genes that encode small molecule transport proteins — there are 591 such genes in *E. coli*, which comprise 13% of the genome¹⁵. A subset of these genes encode proteins that mediate small molecule efflux. Many of these efflux systems, such as AcrAB/TolC¹⁶, are not selective for a specific class of antibiotic, but can rid cells of various toxic compounds, thereby providing innate resistance to antibiotics. Other efflux systems are highly specific and can be triggered by exposure to antibiotics. Unlike the innate resistance efflux proteins, the TetA efflux pump is selective for tetracycline antibiotics¹⁷. Expression of the *tetA* gene is negatively regulated by TetR, a tetracycline-sensitive gene regulator that stringently controls efflux pump expression¹⁸. The evidence for the association of resistance with a loss of fitness is outlined in BOX 1.

Box 1 | What is the fitness cost of antibiotic resistance?

A common assumption, which is supported by experimental evidence, is that mutations that confer antibiotic resistance to an organism reciprocally and negatively impact the microorganisms fitness. Therefore, if the antibiotic is withdrawn, less-fit antibiotic resistant mutants are out-competed by organisms that are susceptible to the antibiotic, thereby diluting antibiotic resistance in the microbial population. Consequently, antibiotic cycling has been recommended as one method of controlling antibiotic resistance. However, in many instances it has been shown that: first, compensatory mutations can restore fitness while maintaining antibiotic resistance; second, some mutations that confer resistance have little or no fitness costs; and third, that some mutations that confer resistance result in increased fitness (reviewed in REFS. 94,95). Therefore, withdrawing the antibiotic might have little effect on reducing resistance levels. Furthermore, recent evidence shows that the capacity of microorganisms to evolve compensatory mutations means that resistance is more likely and can emerge much faster than was previously thought⁹⁶. In the drug-discovery process, a more important measure of the impact of mutations on resistance might not be whether resistant mutants can be identified (for example, by serial passage of a new antibiotic candidate in the laboratory) but whether these mutants have reduced fitness in the host, and whether compensatory mutations are readily isolated.

What about resistance that is conferred by the expression of genes encoding modifying enzymes or efflux pumps? These genes, for example *tetR/tetA*, *ampR/ampC* and *vanRS/vanHAX*, are frequently under strict genetic control, which indicates that unregulated expression is deleterious to the cell. Other resistance genes, for example aminoglycoside resistance genes, are constitutively expressed but their expression can reduce fitness⁹⁷. Because many resistance genes are found on mobile genetic elements, they might be lost from subsequent generations in the absence of antibiotic selection, which would support a policy of antibiotic cycling. However, because most resistance genes on these mobile elements are localized with other resistance genes, co-selection can stabilize the resistance phenotype even in the absence of specific selection. Furthermore, sequencing of bacterial genomes has revealed the presence of numerous cryptic resistance genes that do not seem to affect fitness, however, the impact of these cryptic genes on resistance phenotypes needs to be rigorously evaluated.

In order for antibiotics to be effective, they must bind to their biochemical target(s) with enough saturation to block the target's normal cellular function at a level sufficient to arrest cell growth. This interaction is governed by both thermodynamic and kinetic components. First the antibiotic must bind to the target sufficiently tightly. This thermodynamic interaction is a function of the concentrations of both target and antibiotic and their reciprocal complementarity (K_d). The kinetic component is essential to ensure that the antibiotic can accumulate in the vicinity of the target over time. By controlling antibiotic influx and efflux, the bacteria modulate the kinetic component of antibiotic activity resulting in resistance.

The efflux and influx systems can be co-regulated and connected to a genetic network of auxiliary proteins, for example, this occurs in the *mar* regulon of *E. coli*¹⁹. Exposure to antibiotics results in pleiotropic effects^{20,21} including efflux gene expression, alteration of porin protein content and modulation of the expression of several stress genes. Transcriptional activation of the *mar* locus results in phenotypes that are not selective for specific antibiotics or classes, but that have general consequences for exposure of cells to cytotoxic agents, including resistance to various structurally diverse antibiotics such as fluoroquinolones, tetracyclines and chloramphenicol.

Other resistance systems are more specific. For example, enzymes that inactivate antibiotics such as β -lactamases and aminoglycoside kinases are class (and sometimes compound) specific²². Many of these enzymes draw near to the theoretical kinetic limits for enzyme activity and are so-called perfect enzymes, for which the k_{cat} and K_m approach the limits of diffusion of small molecules in solution. The metabolic bypass mechanism of resistance to the glycopeptide antibiotic vancomycin is

also exquisitely specific^{23,24}. Finally, target modification (of which the vancomycin metabolic bypass system is one example) is perhaps the most specific mechanism of resistance. Mutation(s) in target genes, for example *gyrA* and *parC* in the case of fluoroquinolones, *rpoB* for rifampin and 23S rDNA for linezolid, can overcome the toxic effects of antibiotics.

Furthermore, several mechanisms of resistance to a single antibiotic class can be coordinately regulated in a single pathogen, thereby raising resistance levels. This can result from multiple alterations to a single mechanism, for example heterozygous mutations in multiple copies of the 5–6 23S rDNA genes in *S. aureus* that results in increasing levels of linezolid resistance²⁵. More often though, increased resistance to a specific class of antibiotics is the result of multiple modes of resistance that each contribute to the overall resistance phenotype present in a single organism. For example, *P. aeruginosa* combines enzymatic modification and efflux to resist most aminoglycoside antibiotics²⁶. Of course, expression of multiple antibiotic resistance genes need not be restricted to a single antibiotic class, and indeed it is commonplace for antibiotic resistance plasmids, transposons and integrons to include multiple resistance genes. Control of such combinatorial resistance is a major challenge.

Resistance genes in the pan-microbial genome

The focus of attention of the medical and research community in the past has been on the resistance mechanisms of pathogenic bacteria. However, in many cases these studies provide little information on the origins and sources of antibiotic resistance. A broader view of antibiotic resistance would include resistance genes of pathogenic and non-pathogenic bacteria and even those

genes with the potential to function as resistance genes. This would encompass the full pan-microbial genome — that is, the sum of all microbial genomes. We have proposed that the collection of all the antibiotic resistance genes in microorganisms be known as the antibiotic resistome²⁷ (FIG. 1).

Antibiotic resistance genes from pathogens comprise only a tiny fraction of the resistome. Resistance genes from non-pathogenic bacteria include those from antibiotic producers and cryptic resistance genes. Also included in the resistome are the multiple genes that encode proteins with modest resistance, or antibiotic binding functions, which might evolve into *bona fide* resistance elements, given the appropriate selection pressure. These resistance precursor genes are the ultimate external source of antibiotic resistance.

Genome sequencing endeavours have revealed a plethora of resistance genes that are present in all bacteria. For instance, genes that encode efflux proteins are common to all bacterial genomes. Opportunistic pathogens such as *P. aeruginosa* that are normally found in various environments (soil and fresh water) have a remarkable collection of efflux pumps^{28–30}. These pumps presumably function to provide the maximum flexibility to the organism so that it can exploit diverse environments, to promote pathogenicity, and to modulate cellular differentiation, such as the formation of biofilms³¹.

Many bacteria encode β -lactamases, enzymes that can hydrolytically inactivate β -lactam antibiotics such as the penicillins and cephalosporins³². These genes, such as *ampC* in *E. coli* and its orthologues in other enteric bacteria, are embedded in the chromosomes of

pathogenic and non-pathogenic bacteria and mediate resistance on overexpression. These β -lactamases are often stringently regulated and are inducible by antibiotic exposure for 'just in time' delivery of protection. By contrast, β -lactamases encoded on resistance plasmids, transposons, and integrons found in clinically resistant bacterial isolates, are generally constitutively expressed. Perhaps this is because the host organism inhabits environments in which antibiotics are found in high concentrations and regulated expression of resistance genes offers little advantage.

Another example of chromosomally encoded antibiotic resistance is that of streptogramin resistance (FIG. 2). Type A streptogramins are cyclic polyketide-amino acid hybrids and type B streptogramins are cyclic depsipeptides. These antibiotics synergistically bind to the large subunit of the bacterial ribosome blocking translation. Streptogramins cannot cross the outer membrane of most Gram-negative bacteria and are primarily effective against Gram-positive organisms. Enzymatic resistance to type A streptogramins is catalysed by the Vat (virginiamycin acetyltransferase) enzymes of the left-handed parallel β -helix family of proteins³³ found on various resistance plasmids (R-plasmids) in Gram-positive pathogens (staphylococci and enterococci, for example). A search of bacterial genome sequences reveals several homologous Vat-encoding genes that are widely distributed in non-pathogenic bacteria, including the intrinsically resistant Gram-negative bacteria. A study of one of these orthologous proteins from *Yersinia enterocolitica* has confirmed the predicted ability of Vat enzymes to acetylate type A streptogramins³⁴.

Similarly, *vgb* (virginiamycin resistance gene B), which codes for an enzyme that inactivates type B streptogramins by an unusual lyase mechanism (FIG. 2c), is also present on plasmids of Gram-positive pathogens. Searching for *vgb* homologues in bacterial genomes revealed several genes encoding similar proteins, including *vgb* genes present in several Gram-negative bacteria. Purification of the proteins encoded by *Streptomyces coelicolor* (Gram-positive soil organism) and *Bordetella pertussis* (Gram-negative opportunistic pathogen) *vgb* genes, and analysis of the enzymatic activity of the Vgb proteins, confirmed that these genes were orthologues of the R-plasmid encoded Vgb³⁵. Bacterial genomes are therefore reservoirs of streptogramin resistance genes.

The extended spectrum β -lactamases of the CTX-M family that are presently a clinical scourge³⁶, probably originated in *Kluyvera* species that are widespread in the environment. Genes encoding CTX-M β -lactamases have been found in the chromosomes of *Kluyvera ascorbata*³⁷, *Kluyvera georgiana*³⁸ and *Kluyvera cryocrescens*³⁹ and are the probable progenitors of the clinically associated genes⁴⁰.

Study of the resistome requires not only gene-sequence analyses but also rigorous follow-up experimental validation. A case in point is aminoglycoside resistance. Aminoglycoside antibiotic resistance is frequently the result of modifying enzymes that phosphorylate, acetylate or adenylate the antibiotic scaffold⁴¹. Numerous putative aminoglycoside kinases and acetyltransferases

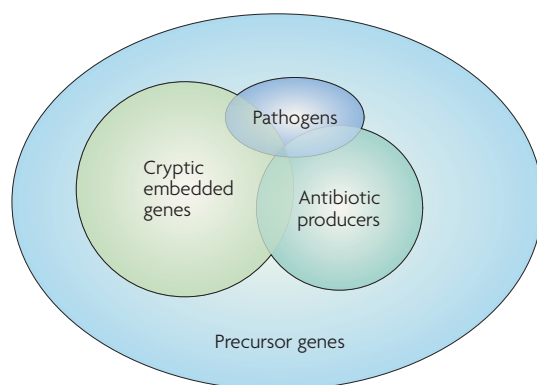


Figure 1 | The antibiotic resistome. The resistome comprises all of the antibiotic resistance genes. It includes resistance elements found in both pathogenic bacteria and antibiotic-producing bacteria, and cryptic resistance genes (which are not necessarily expressed) that are present in bacterial chromosomes. Resistance genes encode proteins that can either be highly specific to classes of antibiotics or can be generalists with broad specificities. The resistome also includes precursor genes that encode proteins with modest antibiotic resistance activity, or affinity to antibiotics, that might evolve into effective resistance genes. Genes that encode resistance genes in antibiotic producers, or that are cryptic can be similar to the genes emerging in pathogenic bacteria; consequently these gene sets can significantly overlap.

Resistome

A collection of all the antibiotic resistance genes and their precursors in pathogenic and non-pathogenic bacteria.

Cryptic resistance gene

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

R-plasmid

A plasmid that is present in bacterial pathogens and environmental microorganisms, and that contains one or more antibiotic resistance genes.

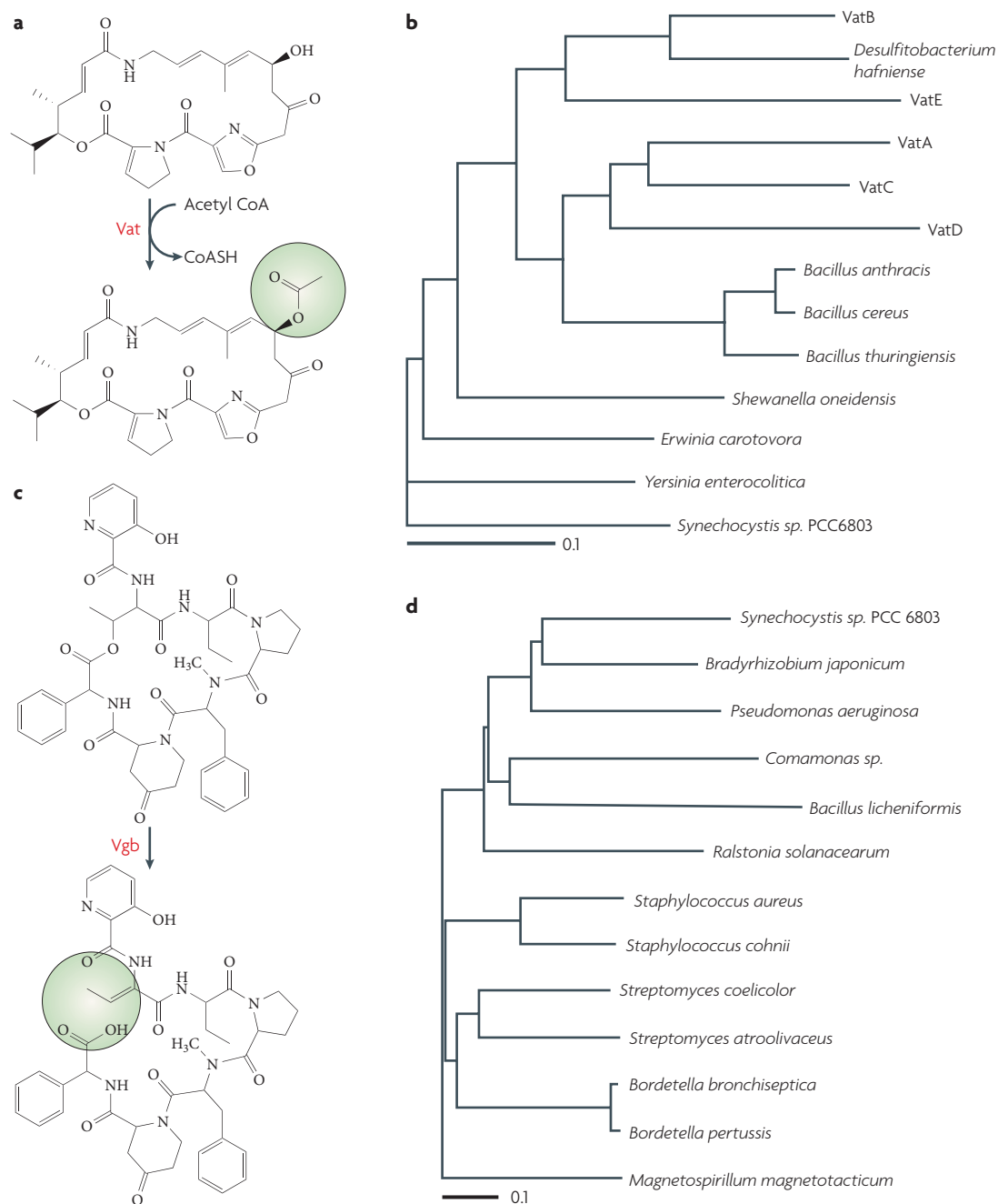


Figure 2 | Streptogramin antibiotics and streptogramin resistance. Resistance to type A streptogramin antibiotics occurs by the acetylation of a crucial hydroxyl group. **a** | Shows the chemical modification of pristinamycin IIA, which is catalysed by Vat enzymes (virginiamycin acetyltransferase). **b** | Homologues and orthologues of the Vat enzymes that are found in clinically resistant bacterial strains are widely distributed in chromosomes of numerous environmental bacterial species. **c** | Type B streptogramin resistance is catalysed by Vgb (virginiamycin resistance gene B) and its orthologues. Cleavage of the cyclic depsipeptide of pristinamycin IA by the enzyme Vgb, an unusual C-O lyase, results in antibiotic resistance. **d** | Orthologous and homologous *vgb* genes are distributed among the genomes of many environmental bacteria, including *Streptomyces coelicolor* and *Bordetella pertussis*. The amino-acid sequence alignments were constructed using Clustal W⁹⁸ and the tree was drawn with NJplot⁹⁹. Note that the trees are not a rigorous phylogenetic analysis, but rather an attempt to convey the sequence relationship among these enzymes.

have been uncovered during bacterial genome-sequence analyses. On the one hand, some of these genes, such as APH(3')-IIb from *P. aeruginosa*⁴² and APH(9) from *Legionella pneumophila*⁴³, have been experimentally characterized as highly efficient aminoglycoside

resistance elements. On the other hand, Rv3817 and Rv325c from *Mycobacterium tuberculosis*, which were annotated as putative aminoglycoside kinases, failed to confer aminoglycoside resistance activity when expressed in *E. coli*⁴⁴. However, purified Rv325c did have modest

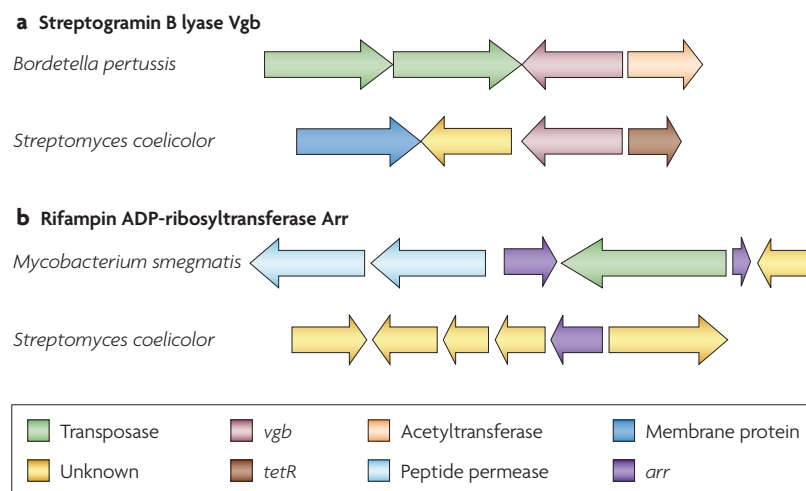


Figure 3 | Genome context of chromosomal antibiotic resistance genes. Bacterial genome sequences have revealed a remarkable number of genes that are predicted to encode antibiotic resistance proteins. Genes encoding proteins that mediate horizontal gene transfer, such as transposases and predicted insertion element DNA sequences, flank many of these resistance genes, indicating previous gene-mobilization events. However many chromosomal antibiotic resistance genes are not linked to such elements, and roles of the associated proteins are generally unknown. This figure shows the genome context of genes encoding protein orthologues of streptogramin B lyase Vgb³⁵ (**a**) and rifampin ADP-ribosyltransferase (**b**; Baysarovich and G.D.W., unpublished data). The *tetR* gene adjacent to the *vgb* orthologue in *S. coelicolor* is predicted to encode a TetR-like transcription regulator. The smaller *arr* gene in the *M. smegmatis* cluster is a truncated version of the full-length gene. Arr, rifampin ADP ribosyltransferase; Vgb, virginiamycin resistance gene B.

in vitro aminoglycoside modifying activity. Rv325c might therefore have the capacity to evolve into a robust resistance element. In the nomenclature of FIGURE 1, we would classify this gene as a resistance precursor.

The revelation that most bacterial genomes include resistance genes and their precursors (even in bacteria that are not normally susceptible to the antibiotic) is not limited to the organisms for which we have genome sequence data. This assertion was put to the test in a screen of ~500 spore-forming soil bacteria collected from various environments against 21 antibiotics²⁷. Remarkably, this study revealed that all sampled bacteria were multidrug resistant. On average, each bacterium was resistant to 7–8 antibiotics. There were no antibiotics for which a resistant organism was not found: old and new antibiotics, natural products and their semi-synthetic derivatives, and synthetic molecules with no relationship to known natural products were all vulnerable to pre-existing bacterial resistance mechanisms.

Two important conclusions can be drawn from this work. First, combinatorial resistance is the default phenotype in these environmental organisms. This parallels the state of resistance in opportunistic pathogens such as *A. baumannii* and *P. aeruginosa*, which are increasingly problematic in the clinic. Multidrug resistance might therefore be the natural state of most microorganisms and we should expect that the next inevitable wave of emerging pathogens will be resistant to multiple

antibiotics. Second, the remarkable scope of resistance to the entire chemical range of drugs used in the study was surprising. It proved facile to identify bacteria resistant to any class of antibiotic, including novel antibiotics that these organisms would not have been exposed to previously. The resistome is therefore comprehensive, adaptable and extensive. The implications for the emergence of resistance in pathogenic bacteria are significant given the potential ability of genes to be mobilized through the pan-microbial genome.

Mobilization of antibiotic resistance elements

How do resistance genes cross species, genus and perhaps even kingdom barriers? Bacteria exchange genetic information either through the direct uptake of DNA (transformation), phage-mediated transduction, through inter-organism contact with DNA exchange (conjugation) or mobilization of DNA within organisms' genomes (transposition). Transposition includes the well known transposons flanked by inverted repeat insertion elements and other elements such as *ISEcp1* involved in mobilization of the *bla*_{CTX-M} gene⁴⁵ and the ISCR elements that only require one insertion element for gene mobilization⁴⁶. Furthermore, resistance gene cassettes can be captured and clustered in integrons and thereafter be mobilized to spread resistance genes to other organisms⁴⁷. In non-pathogenic environmental organisms, plasmids encoding multiple antibiotic resistance genes are prevalent, often clustered with genes that are required for heavy-metal resistance (for examples refer to REFS 48–50).

Evidence for the importance of gene mobilization resulting in antibiotic resistance can be found when scanning the genome contexts of predicted and confirmed resistance genes in bacterial genomes. For example, the *vgb* orthologue in *B. pertussis* is flanked by two predicted transposase genes and an acetyltransferase gene (FIG. 3). The transposase genes are associated with many transposons and the acetyltransferase genes are commonly found in mobile genetic elements. The *B. pertussis* *vgb* gene might therefore be a molecular fossil of a transposition event that occurred prior to speciation, as the gene is present in all strains of *B. pertussis*. Similarly, a rifampin ADP ribosyltransferase (encoded by *arr*) is found in all isolates of *Mycobacterium smegmatis*. This gene is flanked by a predicted transposase as well, suggesting that the gene was transferred prior to speciation by a transposition event (FIG. 3).

The remarkable ability of bacteria to mobilize genes and the selective pressure provided by antibiotics, conspire to facilitate the distribution of antibiotic resistance genes throughout microbial populations. As a result, the resistome expands even in the absence of continuous selection.

Evolution of resistance genes

Where do antibiotic resistance genes come from in the first place? Protein structure and function indicates that resistance proteins share common properties with proteins that have non-resistance functions. Resistance proteins have presumably evolved over time

from ancestral precursors that are either sensitive to, or that have little affinity for, antibiotics into the highly specific enzymes and proteins that plague the healthcare sector today. For the most part, these resistance elements have probably been circulating in bacterial populations for millennia rather than emerging since the golden era of antibiotic discovery 50 years ago.

Antibiotics are ancient. Baltz has estimated that erythromycin, streptomycin and vancomycin biosynthetic pathways emerged over 880, 610 and 240 million years ago, respectively⁵¹. Even the relatively new antibiotic daptomycin is predicted to be at least 30 million years

old! Furthermore, using a structure-based phylogeny, Hall and Barlow estimated that β -lactamases arose over 2 billion years ago, predating the divergence of Gram-positive and Gram-negative bacteria⁵². Given the ability of bacteria to replicate quickly, the ease of horizontal gene transfer, the selective pressure from antibiotic use and the fact that antibiotics predate the dinosaurs (and even the Cambrian explosion) the inevitability of resistance becomes obvious.

The resistance elements frequently encountered on R-plasmids in clinical bacterial isolates are generally highly specific for antibiotic detoxification. These resistance genes are often too sophisticated to have arisen from the gradual evolution of precursors present within the genomes of pathogens in the past few decades. Other highly efficient resistance genes are found in bacteria that produce antibiotics⁵³. Resistance genes in such organisms are generally clustered and co-regulated with antibiotic biosynthesis genes. Antibiotic producing bacteria might therefore be reservoirs of efficient resistance genes.

Vancomycin resistance: an elaborate resistance strategy.

The complex mechanism that gives rise to the glycopeptide antibiotics such as vancomycin is an instructive example of clinical resistance that might originate from antibiotic producing bacteria. Vancomycin binds non-covalently to the *N*-acyl-D-Ala-D-Ala segment of the bacterial cell wall through a network of five hydrogen bonds⁵⁴ (FIG. 4). The D-Ala-D-Ala dipeptide is virtually ubiquitous in cell-wall-containing bacteria. The antibiotic-D-Ala-D-Ala complex forms on the outside of the cell on peptidoglycan precursors such as lipid II, and prevents both transglycosylase-catalysed cell-wall growth and the transpeptidase-mediated inter-strand crosslinking that provides essential rigidity to the cell-wall polymer. The outer membrane of Gram-negative bacteria provides intrinsic protection from glycopeptide antibiotics, but Gram-positive bacteria are susceptible, owing to their exposed peptidoglycan layers.

Glycopeptide antibiotics bind to a ubiquitous cell-wall polymer, and not to a protein target that could be mutated to provide resistance; furthermore, they are effective at the exterior of the cell and are not susceptible to efflux or influx resistance mechanisms. This led to predictions that it was unlikely that resistance to vancomycin and other glycopeptides would readily occur. Nevertheless, four decades after its discovery, and commensurate with increased clinical use⁵⁵, isolates of VRE were reported⁵⁶ and are now a major cause of nosocomial infection in many hospitals. Moreover, vancomycin resistance has also recently emerged in the virulent clinical pathogen *S. aureus* (VRSA)^{57,58}, expanding the scope of this problem.

The mechanism of resistance in VRE and VRSA is elegant and requires a two-component regulatory system, VanR and VanS, and three enzymes, VanH, VanA and VanX (Fig 4). The two-component system senses the presence of vancomycin and activates the expression of the *vanH*, *vanA* and *vanX* genes. Extensive genetic and biochemical study by the groups of Patrice Courvalin and Christopher Walsh have identified the essential

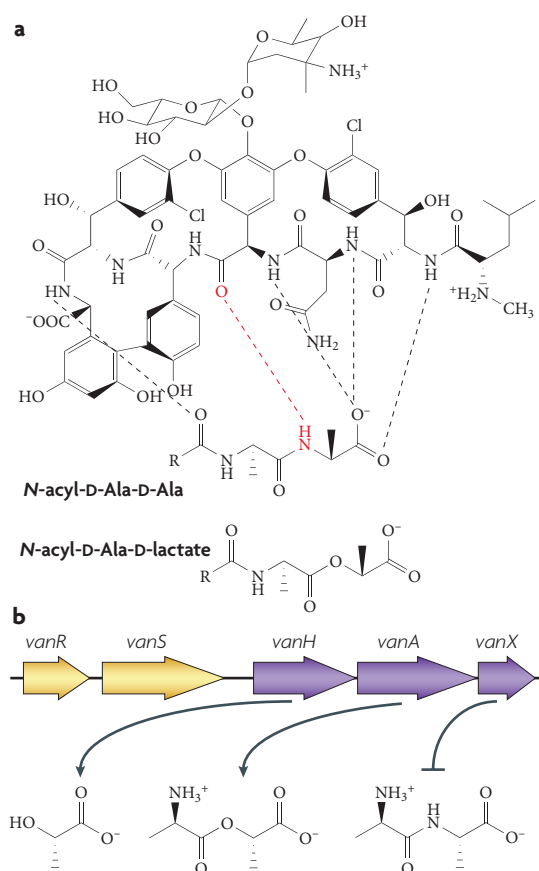


Figure 4 | Vancomycin resistance: an elegant mechanism of antibiotic evasion. **a** | Vancomycin binds to the *N*-acyl-D-Ala-D-Ala dipeptide that caps the peptide portion of the bacterial peptidoglycan through five hydrogen bonds. Vancomycin-resistant bacteria synthesize peptidoglycan molecules that terminate in *N*-acyl-D-Ala-D-Lactate, which removes a key hydrogen bond interaction, resulting in a 10^3 -fold decrease in affinity of the antibiotic for the cell wall. **b** | The D-Ala-D-Lactate depsipeptide dominates in resistant bacteria through the aegis of a five gene cluster. All five genes are required for inducible vancomycin resistance. A two-component regulatory system, VanR–VanS positively regulates the expression of VanH, VanA and VanX in response to antibiotic exposure. VanH catalyses the synthesis of D-Lactate and VanA ligates this D-Lactate to D-Ala. VanX is a peptidase specific for D-Ala-D-Ala that continues to be synthesized *in vivo* by endogenous D-Ala-D-Ala ligase.

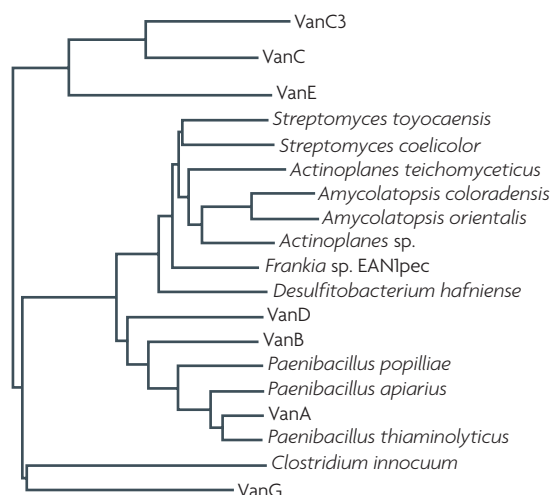


Figure 5 | Vancomycin-resistance genes are widespread in the environment. Homologues of VanA were identified by a BLAST search, the alignment was constructed with ClustalW⁹⁸ and the tree was drawn with Njplot⁹⁹. Known D-Ala-D-Ala ligases were omitted from the analysis. Note that the trees are not a rigorous phylogenetic analysis, but rather are an attempt to convey sequence relationship among these enzymes.

roles of each protein in drug resistance^{24,59,60}. VanH is an α -ketoacid reductase that converts pyruvate to D-lactate. VanA is a homologue of the essential D-Ala-D-Ala ligases that produce this essential cell-wall component. However, VanA prefers the D-Ala isostere D-Lactate as a substrate and generates the ester (depsipeptide) D-Ala-D-Lactate rather than the D-Ala-D-Ala peptide. Finally, VanX is a highly specific Zn²⁺-dependent dipeptidase that depletes the cellular pools of D-Ala-D-Ala that continue to be constitutively produced by normal cell-wall metabolism. As a result, the peptidoglycan of the vancomycin-resistant cell incorporates the ester D-Ala-D-Lactate rather than the peptide D-Ala-D-Ala. This seemingly minor substitution of an oxygen for a nitrogen results in the loss of a hydrogen bond donor and electronic clashes between acyl-D-Ala-D-Lactate and vancomycin. Both of these factors contribute to a 1000-fold decrease in affinity between the antibiotic and its ligand, culminating in high-level drug resistance⁶¹.

When this new resistance mechanism emerged in the clinic in the late 1980s there seemed to be no precedent. Where did this intricate mechanism originate from? Subsequent sequencing of glycopeptide antibiotic biosynthetic gene clusters revealed similar *vanHAX* clusters^{62,63} and biochemical characterization of the gene products confirmed this assignment^{64–66}. All glycopeptide producing bacteria seem to use the same resistance mechanism. The arrangement of genes is conserved between VRE and vancomycin-producing bacteria, as is a short overlap between the *vanH* and *vanA* genes, which might indicate that antibiotic producers were the origins of glycopeptide resistance. Despite the ‘smoking gun’ connection between vancomycin resistance in the clinic and vancomycin resistance in the environment,

there is a significant discrepancy in the GC content of the resistance genes found in VRE (~40%) and vancomycin-producing bacteria (~70%). The recent direct transfer of resistance genes from environmental producers to clinical pathogens is unlikely, and given the predicted age of glycopeptides (240 million years old), there has been plenty of time for drift of GC content to the present levels found in VRE. At present there is an incomplete ‘fossil record’ of the evolution of vancomycin resistance and the movement of these genes from the environment to the clinic. In other words, there are missing links to be discovered. The publication of increasing numbers of bacterial genome sequences is likely to shed light on some of the gaps in our knowledge of this process.

A survey of bacterial genome sequences and the literature^{67,68} shows that other environmental bacteria — including members of the actinomycete class such as *Frankia*, and *Streptomyces* and bacilli such as *Paenibacillus* — that are not known to produce glycopeptides also harbour the *vanHAX* resistance gene cluster (Fig. 5). In common with the examples of Vgb and Arr, analysis of flanking genetic sequences has, in many cases, identified gene-mobilizing elements that might implicate horizontal gene transfer rather than *de novo* evolution of glycopeptide resistance genes.

Aminoglycoside inactivating enzymes. The notion that antibiotic producers are potential sources of clinical resistance is not a new concept. Benveniste and Davies highlighted the functional relationship between aminoglycoside resistance in clinically resistant organisms and aminoglycoside producers more than 30 years ago⁶⁹. In one of the best-studied systems, an aminoglycoside kinase (APH) modifies the antibiotic streptomycin during biosynthesis, which protects the organism from suicide, and a cognate phosphatase cleaves the protecting modifying group after the release of the antibiotic outside the cell⁷⁰. Orthologues of these protective kinases are commonly located on transposons, integrons and R-plasmids, and are widely distributed among clinically problematic pathogenic bacteria, indicating a common origin.

Where did APHs originate? Extensive biochemical studies have shown that APHs share three-dimensional structure, enzymatic mechanism, sensitivity to inhibitors and substrate specificity with Ser/Thr/Tyr protein kinases^{71–74}. Once thought to be exclusively found in eukaryotes, in which they have central roles in signal transduction, this class of kinase is now known to be widely distributed in bacteria^{75,76}. Genome sequencing of actinomycetes, which include many antibiotic producing bacteria, have revealed numerous genes encoding Ser/Thr/Tyr protein kinases^{77,78}. This provides a plausible environment for the evolution of APHs from an ancestor with a protein kinase fold with antibiotic modifying activity. This hypothesis is supported by the observation that APHs have measurable Ser-protein-kinase activity⁷².

Similarly, aminoglycoside resistance can also frequently occur by acetyltransferases (AACs), which modify key amino groups that are required for biological activity. Structural and biochemical analysis of AACs has shown that these proteins are members

of the GCN5-related superfamily of acyltransferases^{79–82}. Bacterial genome sequencing has revealed hundreds of members of this family that currently have no known function. This protein scaffold seems to be particularly malleable, with functions of member enzymes ranging from small-molecule acetylation (for example, AACs) to protein modification (for example, histone acetyltransferases)⁸³. Purified AACs have also been shown to have protein acetylating activity^{80,82}, reinforcing the biochemical connection among members of the family. Perhaps AACs have evolved from GCN5 proteins that have different functions, or perhaps AACs are acetyltransferases that have fortuitous antibiotic inactivation activity. For example, Blanchard and colleagues hypothesized that the chromosomally encoded AAC(2′)-Ic, which confers resistance to a broad range of aminoglycosides in *M. tuberculosis*, might be involved in mycothiol biosynthesis⁷⁹.

β -Lactamases. There is agreement that serine β -lactamases probably evolved from penicillin-binding-proteins, such as the peptidoglycan modifying DD-peptidases^{32,84}. The structures and biochemical mechanisms of these proteins are conserved and are widely distributed among bacteria. In this case, evolutionary pressure has selected for modification of the antibiotic target into a form that degrades the antimicrobial agents.

A model for the evolution of antibiotic resistance. A consensus model that has emerged from this growing body of biochemical, protein structural and genomic data is that antibiotic resistance proteins evolve from proteins with alternative biochemical functions that function as precursors to resistance elements (FIG. 6). Some of these precursor proteins might have modest and fortuitous antibiotic resistance functions or other affinities for the antibiotic that, in the face of selective pressure, evolves into a robust resistance mechanism. Exposure to the chemical diversity of antimicrobial compounds over millennia has spurred reciprocal genetic countermeasures to yield the highly efficient resistance genes of today.

This is also a contemporary and ongoing process. Microorganisms, plants and animals are continually expanding the chemical diversity of molecules they produce, through the evolution of secondary metabolite biosynthetic pathways. Humans are also developing new antimicrobial chemical scaffolds, but the reciprocal resistome genetic diversity can be used to address these new molecules. Hooper's group recently reported the remarkable evolution of an aminoglycoside acetyltransferase into a ciprofloxacin resistance enzyme⁸⁵. This unprecedented enzymatic activity with a totally synthetic antibiotic substrate was probably selected in the clinic and persuasively demonstrates that even synthetic antibiotics are not impervious to the stunning ability of microorganisms to sample genetic diversity to defend themselves.

Because environmental organisms that produce secondary metabolites such as antibiotics must also evolve mechanisms to protect themselves from the toxic activity of these molecules, producer organisms are 'hot spots' for the evolution of antibiotic resistance. Similarly, their microbial neighbours (in specific niches) must co-evolve coping strategies to compete for resources. This can occur either through the evolution of resistance mechanisms in the microbial neighbour, or by horizontal gene transfer of resistance genes from other bacterial species.

Recently, the finding that some bacteria respond to the presence of an antibiotic by increasing their mutation frequency has led to the proposal of another mechanism for the rapid evolution of resistance in the face of chemical threats^{86,87}. Antibiotics might therefore have a central role in the evolution of resistance other than simply providing selective pressure⁸⁸. Furthermore, it is clear that the cytotoxic effects of antibiotics might not be their primary role. Sub-lethal concentrations of antibiotic trigger a myriad of genetic effects, including modulation of gene transcription in non-obvious ways^{20,21,89}. For example, the activity of ~5% of gene promoters in

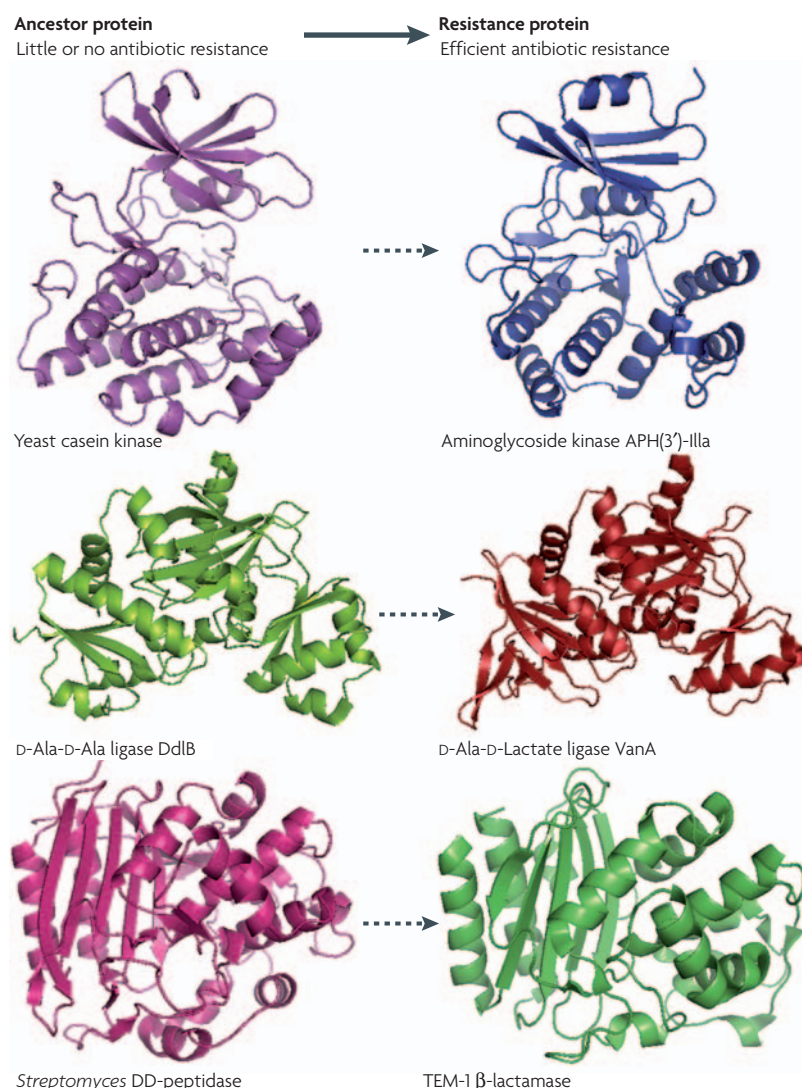


Figure 6 | Evolution of antibiotic resistance proteins. Protein structure and mechanism studies reveal that antibiotic resistance proteins are related to proteins with little or no antibiotic affinity. The dotted arrows indicate that proteins might either be the immediate precursors of resistance proteins, or that they might share common ancestry with resistance proteins.

Salmonella enterica serovar Typhimurium is modulated by exposure to sub-lethal concentrations of rifampin or erythromycin²¹. The true function of some resistance genes and their associated proteins therefore might not be to detoxify antibiotics, but to receive, or otherwise modulate, chemical signals. This is a fascinating new area of research that requires a more comprehensive understanding of the microbial ecology of antibiotic producers and their neighbours than we currently have.

Chemical diversity spurs resistome diversity

The clinics, which are polluted with antibiotics, are 'war zones' in which there is an ongoing struggle between infectious organisms and patients, with healthcare professionals trying to intervene as peacekeepers. The fact that this is an enduring battle without an easy solution can now be viewed in light of the microbial genomes and metabolism outside the clinic. Environmental microorganisms are successful chemists that continue to evolve new biosynthetic pathways that produce complex bioactive natural products while maintaining evolutionarily successful ones that arose millions of years ago. Genome sequencing of environmental organisms has begun to reveal the depth of the chemical diversity available to microorganisms (for example, the number of predicted natural product biosynthetic clusters is 22 in *S. coelicolor*⁷⁷, and 30 in *Streptomyces avermitilis*⁷⁸). Furthermore, the modular nature of the polyketide and non-ribosomal peptide synthesis biosynthetic machineries assures that the combinatorial expansion of natural product synthesis is essentially limitless⁹⁰.

In the face of this chemical diversity is the reciprocal genetic diversity that interacts with these molecular signals. Most chemicals produced by microorganisms are not cytotoxic, and even those that are toxic require concentrations that might exceed the levels found in natural environments⁹¹. However, the observed density of resistance mechanisms in the soil²⁷, including the data produced by recent metagenomic studies⁹², suggests that many of these natural products function as antibiotics, or at least that they trigger a molecular response by bacteria

that is equivalent to resistance. Therefore, it seems that organisms that have evolved under a constant barrage of increasingly complex chemical diversity are probably the source of many antibiotic resistance genes, and comprise the bulk of the resistome.

What is the impact of the resistome concept on modern medicine? The evidence is clear that environmental bacteria are often more intrinsically drug resistant than the commensal organisms that are the main causes of infectious disease. Furthermore, the mechanisms of resistance to many antibiotics in clinical isolates probably have their origins in the environmental resistome. The widespread use of antibiotics over the past 60 years has provided the requisite conditions to mobilize the highly efficient resistance genes that circulate in the environmental resistome into pathogenic bacteria. It is therefore vital that the use of antibiotics is viewed in the context of a resistome that can respond quickly and inevitably to the use of antimicrobial agents. Therefore antibiotics must be used judiciously to extend the lifetime of these drugs as misuse will inevitably result in resistance.

An interesting question that emerges from this analysis is, if resistance is inevitable, then why is resistance not more widespread? For example, despite decades of clinical use, resistance to the β -lactam antibiotics has not yet emerged in group A streptococci. This question exposes our lack of understanding of the rules of bacterial antibiotic resistance, namely its emergence and evolution. Understanding these rules and their underlying molecular bases will help us to develop the next generation(s) of antibiotics. Moreover, the breadth of the resistome, combined with a growing understanding of the redundancy of resistance mechanisms, indicates that new paradigms in drug discovery and development must be expanded to lessen the impact of resistance to future antibiotics. Systematic sampling of multidrug combinations⁹³, strategic targeting of resistance mechanisms and blocking antibiotic-induced mutation⁸⁶ are some of the options that could extend the potency of antibiotics in the face of the resistome.

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Competing interests statement

The author declares no competing financial interests.

DATABASES

The following terms in this article are linked online to:

Entrez Genome Project: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj>
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