Available online at www.sciencedirect.com

SciVerse ScienceDirect

Current Opinion in Microbiology

Context matters — the complex interplay between resistome genotypes and resistance phenotypes

Gautam Dantas[1,2] and Morten OA Sommer[3,4]

Application of metagenomic functional selections to study antibiotic resistance genes is revealing a highly diverse and complex network of genetic exchange between bacterial pathogens and environmental reservoirs, which likely contributes significantly to increasing resistance levels in pathogens. In some cases, clinically relevant resistance genes have been acquired from organisms where their native function is not antibiotic resistance, and which may not even confer a resistance phenotype in their native context. In this review, we attempt to distinguish the resistance phenotype from the resistome genotype, and we highlight examples of genes and their hosts where this distinction becomes important in order to understand the relevance of environmental niches that contribute most to clinical problems associated with antibiotic resistance.

Addresses
[1] Center for Genome Sciences & Systems Biology, Washington University School of Medicine, St Louis, MO, USA
[2] Department of Pathology & Immunology, Washington University School of Medicine, St Louis, MO, USA
[3] Department of Systems Biology, Technical University of Denmark, DK-2800 Lyngby, Denmark
[4] Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2970 Hørsholm, Denmark

Corresponding authors: Dantas, Gautam (dantas@wustl.edu) and Sommer, Morten OA (msom@bio.dtu.dk)

Current Opinion in Microbiology 2012, **15**:577–582

This review comes from a themed issue on **Antimicrobials**

Edited by **Didier Mazel** and **Shahriar Mobashery**

For a complete overview see the Issue and the Editorial

Available online 3rd September 2012

1369-5274/$ – see front matter,

http://dx.doi.org/10.1016/j.mib.2012.07.004

Introduction

Any gene (or genes) which enables a microorganism to tolerate or resist antibiotics at concentrations which would inhibit the growth of other organisms can be considered as an antibiotic resistance gene (RG). Drug-resistant pathogenic bacteria are continuously identified in both clinical and community settings whose phenotypic resistance can be attributed to genes acquired from another organism through horizontal gene transfer (HGT) via conjugation, transformation, or transduction [1,2,3•,4]. The high concentrations of antibiotics used in clinical and agricultural settings likely provide strong selective pressure favoring such RG exchange between pathogens and microbes from virtually any habitat [1,5,6,7••,8,9,10•,11]. Indeed, there is clear evidence supporting recent RG exchange between pathogens and the commensal microbiota of humans [5–8] and animals [9]. In contrast, there is a surprisingly limited number of examples supporting such exchange between human pathogens and the diverse and ancient resistomes of microbial communities associated with the broader environment, such as soils and water [1,10•,11]. In this context, it is important to recognize that the resistance activity of acquired RGs in pathogens need not reflect the 'normal' function of these genes in their native host contexts — indeed, the acquired RG in the pathogen may not natively encode any antibiotic tolerance function in the donor organism [10•,12]. This can significantly confound our ability to estimate and correlate the relative importance of phenotypic resistance in potential donor bacteria versus their complement of genes which can encode resistance when transferred to pathogenic recipients. We argue that functional classification of RGs is critically dependent on their specific genomic and genetic context and is not a simple case of associating resistance or susceptibility phenotypes of putative donors to the likelihood of resistance genotype accessibility by pathogenic recipients. This idea has begun to receive increasing attention as a consequence of culture-independent metagenomic investigations of resistomes accessible to pathogens [1,7••,13]. As a result it is important to understand and clarify the difference between the phenotypic resistance and the accessible genotypic resistome of any microbial community of interest.

Antibiotic resistance and the antibiotic resistome

Definitions

Antibiotic resistance: A phenotype, which is related to the ability of an organism to grow in the presence of an antibiotic.

Antibiotic resistome: A collection of genes, that is capable of conferring resistance towards antibiotics when expressed in a susceptible organism.

Silent resistance gene: A gene, which does not confer resistance to its native host, yet is capable of conferring resistance when expressed in other hosts

Antibiotic resistance can result from the expression of a specific resistance gene as well as from the absence or reduced access of the drug to its target [14]. Absence or reduced access to the drug target is sometimes referred to

as intrinsic resistance, since it is a result of specific physiological characteristics of the organism [10•,12]. An example of this is vancomycin tolerance of *Escherichia coli* due to the reduced access of vancomycin to the nascent peptidoglycan chains in Gram-negative organisms resulting from the low permeability of the outer membrane [15]. In some cases a resistance phenotype is dependent on the specific growth environment of the organism. For example, drugs such as sulfonamides that inhibit folate biosynthesis can be bypassed if organisms have the ability to import folate precursors from the environment [16]; however, if folate precursors are not available in sufficient quantities from the environment these same organisms will not be able to survive. Similarly an organism may be susceptible to a specific antibiotic when growing in planktonic culture, whereas it is resistant when growing in a structured biofilm [17]. Effective phenotypic resistance can stem from expression of a resistance gene or as a result of the specific cellular physiology. Either way, the organism capable of growing in the presence of antibiotics is resistant, yet the different causes of resistance have a strong impact on the ability of a resistant organism to contribute to the dissemination of antibiotic resistance to other organisms.

The antibiotic resistome consists of all the genes or pathways which are capable of conferring resistance when expressed in a specific host. The resistome of a specific community will contain genes which can confer resistance to large groups of organisms, such as the ermB gene which has been identified across Gram-positive and Gram-negative species [3•], as well as genes that confer resistance only when expressed in specific genera. However, in absence of experimental verification in a variety of host organisms, the spectrum of organisms in which a resistance gene can cause resistance is difficult to assess from its sequence alone. The antibiotic resistome can be defined at multiple levels of complexity, including: the resistance genes within a specific plasmid or strain [18], a defined microbial community [7••,19] or an entire environmental niche [7••,20••].

While the primary function of a resistance gene in its original host need not necessarily be resistance towards antibiotics, it is expected that most genes which constitute a transferable resistome can also confer resistance to their native host organism in the presence of high concentrations of antibiotic [10•,12]. However, this may not always be the case — an organism may contribute to the resistome without itself being resistant, for instance, by encoding a strongly regulated gene that only confers resistance when overexpressed. Such genes constitute silent resistance genes that only confer resistance when removed from their native context. An example of how changing genetic context dramatically influences the clinical impact of an RG is that of plasmid mobilization of chromosomally mediated β-lactamases. For example, AmpC β-lactamases are encoded on the chromosomes of many of the Enterobacteriaceae and a few other organisms, but are often poorly expressed, leading to little to no phenotypic resistance [21]. However, AmpC dissemination and activity is dramatically increased when they are mobilized into epidemic plasmids [22] or alternatively when clinical isolates harboring chromosomal enzymes gain mutations in AmpC promoters or attenuators in response to prolonged β-lactam exposure, leading to substantial AmpC overexpression [23,24••].

While the antibiotic resistance levels of members of a microbial community and its encoded antibiotic resistome are related, there is no direct map between them. Consider as an example a microbial community made up of only Gram-negative bacteria. Such a community would have high levels of resistance to vancomycin due to reduced access to the drug target resulting from the presence of an outer membrane. However, the resistome of this microbial community would not contain vancomycin resistance operons, since the physiological feature mediating the resistance, the outer membrane, is encoded by several tightly regulated pathways and thus would be virtually impossible to transfer to a susceptible Gram-positive bacterium. Conversely, if these Gram-negative bacteria only encoded tightly regulated AmpC β-lactamases, the community would have low levels of resistance towards cephalosporins due to the low expression of the AmpC genes [21]. Yet, the resistome of this community would contain cephalosporin resistance genes, since the unregulated AmpC gene removed from its native context could confer resistance to a previously susceptible organism [21]. If, for example, the objective of characterizing a resistome is to design the optimal treatment of a polymicrobial chronic lung infection, the antibiotic resistance levels (i.e. phenotypes) of the organisms are most relevant. Instead, if the objective is to understand how such a community may contribute to resistance in human pathogens through horizontal gene transfer, the antibiotic resistome (i.e. genotype) is most relevant.

Repurposing environmental genes: the 'original' resistome

Environmental microbes are increasingly accepted as the evolutionary origin of antibiotic resistance genes [1,10•,12,25•]. A compelling theory termed the 'producer hypothesis' postulates that horizontally transferred antibiotic resistance genes in human pathogens originate from organisms that produce these same antibiotics in the environment, such as the soil dwelling Actinomycetes [12,26••]. From a simple self-immunity perspective, antibiotic producers must harbor resistance genes to prevent the inhibitory activity of their antibiotic products to be turned on themselves, and hence resistance genes are likely as old as microbial antibiotic production. Since antibiotic biosynthesis and degradation enzymes are capable of many specific chemical modifications of these

compounds [27,28], they might also be repurposed to catalyze transformations in alternate genomic contexts that modulate or ablate their antimicrobial activity. In support of the producer origin of resistance genes, Davies and colleagues demonstrated almost 40 years ago that the biochemical activity of aminoglycoside resistance enzymes encoded by producers was identical to those found in pathogens [26••]. In 2006, D'Costa *et al.* demonstrated that randomly sampled soil isolates of the producer Streptomycete genus were multidrug resistant; of note the approximately 400 isolates were resistant to 7–8 antimicrobials on average, and one microbe in the set was resistant to as many as 15 compounds, despite no specific selection for resistance during isolation [29•]. The genes underlying these multidrug resistance phenotypes are likely to represent a substantial resistome which may be accessible to pathogens [30].

One of the clearest examples of resistance genes originating from producer self-immunity comes from tetracycline biosynthesis, wherein the nontoxic tetracycline precursor anhydrotetracycline induces expression of the TetA efflux pump to enable 'just-in-time' detoxification of the antibiotic product [31]. Transfer of this self-immunity pump to nonproducer organisms enables them to resist tetracycline [32]. Interestingly, the natural decay of tetracycline to anhydrotetracycline can invert the selective advantage of cohabiting resistant strains over susceptible ones, because of the fitness cost of constitutive TetA expression in the resistant strains through anhydrotetracycline induction [33]. This highlights the complex ecological interplay between antibiotic production, degradation, and resistance. Actinomycete production of antibiotics in the soil has also likely provided selective pressure for nonproducer neighbor organisms to evolve resistance mechanisms, enabling them to share and compete for the ecological niches inhabited by the Actinomycetes [12]. Indeed, recent metagenomic sequencing of 30,000-year-old Beringian permafrost sediments identified a diverse set of genes encoding resistance to β-lactam, tetracycline, and glycopeptide antibiotics [25•], clearly demonstrating that environmental resistomes predate modern clinical anthropogenic antibiotic use.

As in the above example of careful TetA induction during tetracycline biosynthesis [31], many putative RGs of environmental origin are silent resistance genes that are stoichiometrically regulated in their native contexts to have limited or low expression, because they have evolved to function in a coordinated network [10•]. Such putative RGs need not be limited only to biosynthetic clusters in antibiotic producers. Antibiotics target critical and highly conserved metabolic, structural, or regulatory bacterial components [14], and hence most bacteria would be expected to encode gene products which modulate various aspects of the same components. Any such gene product which can interfere with antibiotic activity, for instance by competitive interference with target binding [34], is a putative RG. However, these genes may not encode phenotypic resistance in their native hosts simply due to regulated low activity levels which cannot overcome antibiotic action. However, if these genes are mobilized into a new genetic or genomic context, for instance by HGT into a pathogen, they may become decoupled from their regulatory interactions and may now serve as RGs. This is especially true if they are mobilized, by chance, downstream of strong promoters or ribosomal binding sites, as is observed in modular drug-resistance clusters in transposons and integrons [34–38].

Probably the most intuitive case of 'repurposing' a gene evolved for another function for antibiotic resistance is that of efflux pumps — protein complexes responsible for actively pumping chemicals out of the cell through proton motive force or ATP hydrolysis [39]. For example, resistance/nodulation/cell division (RND) efflux pumps have been identified in all kingdoms of living organisms, where they function as transporters of hydrophobic proteins required for nodulation and cell division, and are also critical for heavy metal efflux [40]. However, when genes in this superfamily such as CmeB, AcrB, and Mex are expressed at high levels in pathogenic isolates of *Campylobacter jejuni*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and others, they are capable of exporting multiple antibiotics and become *de facto* RGs [39,40].

Genetic evidence for exchange of resistance genes between the environment and clinical pathogens

The best current support for recent transfer of an environmental resistance determinant to pathogens comes from the perfect nucleotide identity observed between the class-A CTX-M extended-spectrum β-lactamases (ESBL) encoded on the genomes of free-living environmental *Kluyvera* spp. isolates and those rapidly disseminating on plasmids amongst major global pathogens [41,42••,43]. The only other compelling example of a recent acquisition of RGs by pathogens from an environmental source is the quinolone resistance gene Qnr [44,45•]. It was first identified on a broad-host range conjugative plasmid from a ciprofloxacin-resistant *Klebsiella pneumoniae* isolate [44], and subsequently confirmed to belong to a widespread family of DNA-binding proteins, and localized in the genomes of a variety of rarely pathogenic waterborne species, mainly *Aeromonas* spp., *Vibrio* spp., and *Shewanella* spp. [11,45•,46]. The identification of the qnrS2 variant of this gene in a mobilizable IncQ-related plasmid (pGNB2) from treated wastewater implicates human-contaminated aquatic environments as a likely site for RG exchange between the clinic and the environment [47]. Interestingly, the *Kluyvera* spp. CTX-M β-lactamase and the *Shewanella* spp. Qnr resistance protein were the only examples for

which nucleotide identities were high enough to suggest recent exchange between environment and the clinic. Of note, while antibiotic producer soil Actinomycetes have been clearly demonstrated to be phenotypically multidrug resistant [29•,30] and to express resistance proteins with very similar mechanisms to those found in pathogens [26••], their resistance genes have thus far been found to be highly sequence divergent from those of pathogens [48]. Therefore, the 'producer hypothesis' question, namely whether the resistome of antibiotic producers are in current exchange with the resistome of clinical pathogens, remains unresolved.

There are compelling reasons to expect that anthropogenic practices have led to numerous recent and ongoing resistome exchanges between the environment and clinic, including: firstly, the continued contamination of aquatic and soil environments by high quantities of antibiotics [36•], resistant microbes [27,49–51], and resistance genes [47,52,53]; secondly, the overwhelming use of antibiotics in rearing livestock [50,54] and plant agriculture [55]; thirdly, the escalating levels of antibiotic resistance in the soil over the past century [56•]; and finally, the high mobility of resistance genes [1,57]. The paucity of genetic evidence to date in support of this exchange between the environment and clinic may reflect an under sampling of environmental resistomes, rather than the likelihood that these exchanges have not occurred. This under sampling can at least partially be attributed to limitations of the two primary techniques used to characterize resistant microbes and their presumed resistomes — firstly, culture-based approaches [25•,29•] and secondly, PCR screens [22]. Culture-based approaches are effectively guaranteed to only sample a minority of the environmental resistome because a majority of environmental organisms are recalcitrant to culturing using standard techniques, and these communities are immensely diverse and are composed of species of dramatically varied relative abundances [58,59]. Additionally, as discussed earlier, firstly, phenotypic resistance of a cultured isolate does not necessarily predict whether the resistance is encoded by a transferable gene [10•], or secondly, whether a susceptible isolate may still contain genes which only express their resistance function in the genomic context of a new host, for example, due to loss of native regulation [21]. PCR screens using primers complementary to known RGs can circumvent the requirement for culture [22], but their exquisite specificity is also their main limitation — they can only detect known RG sequences, and they require expression-cloning to explicitly verify the predicted function of positive amplicons. In recent years, a complementary approach for resistome characterization has been developed and termed as 'metagenomic functional selections' [1,7••,13,20••,59], which addresses these limitations and is ideally suited to identify any gene which can function as a resistance gene in a host of interest (e.g. pathogen), independent of its function in its native host context.

In metagenomic functional selections, complete microbial genomic DNA is extracted from a microbial community without the need for initial culturing, cloned in-bulk into an expression vector, and transformed into a genetically tractable host bacterium. The resulting library of metagenomic transformants is then subjected to antibiotic selection at concentrations inhibitory to the untransformed host, and the captured metagenomic DNA from surviving transformants can be amplified and sequenced to identify the acquired resistance gene(s). Robust methods have been optimized to clone metagenomic DNA fragments of varying size, ranging from one to a few genes (2–5 kilobases, KB) [7••,13,20••] to operon and mini-chromosome sized gene clusters (40–100 KB) [20••,59,60]. The smaller insert cloning methods are well suited to identify genes capable of encoding acquirable resistance because a majority of known resistance mechanisms can be encoded by one to a few genes [14], and smaller clusters of genes are easier to mobilize through HGT simply due to entropic reasons. Indeed, small-insert functional metagenomics has been extremely successful at identifying large numbers of novel resistance genes, from essentially any microbial habitat sampled [1,7••,13,20••,59,61••]. This includes studies of soils and aquatic environments spanning a range of known direct anthropogenic perturbations (e.g. pristine soils and waterways with minimal human impact versus similar environments heavily contaminated by antibiotic residues and human or animal associated microbes including known pathogens). While such studies have established clear genetic evidence for recent RG transfer between pathogens and human commensal microbiota [7••,8], they have notably thus far been unsuccessful at uncovering evidence for such recent transfer between nonpathogenic environmental microbes and human pathogens, similar to results from culture-based or PCR-based assays [12,48]. Again, this might come down to a sampling issue — standard metagenomic functional selections sample only the most abundant members of a community. It is estimated that 10 terabase metagenomic libraries are required to adequately represent genomes below 1% abundance in any given soil metagenome — orders of magnitude larger than any metagenomic library created to date [59].

Rather than rely on any single approach, we recently speculated that identifying the 'missing genetic link' that explicitly ties environmental resistomes to modern pathogenic resistomes may require a marriage of the above methods, perhaps by initial enrichment of specific subsets of microbial communities by culturing or physical separation methods, followed by functional metagenomic characterization. For instance, selective media conditions have been shown to robustly enrich for multidrug resistant bacteria from the producer Streptomycete genus from different soils [29•], and similar approaches may be employed to enrich for other metabolic or phylogenetic subsets, followed by resistome interrogation. Employing this strategy, we recently enriched for multidrug resistant

Proteobacteria from various US soils and subsequently performed functional selections for antibiotic resistance using a metagenomic library constructed from this highly resistant soil microbial community [61••]. We discovered seven RGs from this soil resistome with perfect nucleotide identity to numerous globally distributed clinical pathogens, representing all major known mechanisms of resistance. We determined that these RGs originated from two traditionally nonpathogenic environmental organisms (*Pseudomonas fluorescens* and *Ochrobactrum anthropi*), and in multiple instances were co-localized within multidrug resistance cassettes flanked by mobilization sequences promoting lateral gene transfer. These results demonstrate that the soil resistome is indeed in recent exchange with human pathogens and highlight the importance of continued work to identify how particular environments, or anthropogenic practices, influence the likelihood of RG exchange between these resistance reservoirs. We expect that the continued integration of improved cultivation based methods [19,29•,30], metagenomic functional selections [1,7••,13,19,20••,59,61••] and DNA sequencing [19,22,25•,61••] will begin to fill the gaps in our current understanding about the dissemination of resistance genes across different environmental niches and afford a more complete picture of resistance gene exchange on our planet.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J: **Call of the wild: antibiotic resistance genes in natural environments**. *Nat Rev Microbiol* 2010, **8**:251-259.

2. Lester CH, Frimodt-Moller N, Sorensen TL, Monnet DL, Hammerum AM: **In vivo transfer of the vanA resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers**. *Antimicrob Agents Chemother* 2006, **50**:596-599.

3. • Shoemaker NB, Vlamakis H, Hayes K, Salyers AA: **Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among Bacteroides and other genera in the human colon**. *Appl Environ Microbiol* 2001, **67**:561-568.

Key paper demonstrating that resistance genes identical at the nucleotide level are shared between phylogenetically distinct species in the human gut providing strong evidence for horizontal gene transfer of resistance genes in the human microbiota.

4. de la Cruz F, Davies J: **Horizontal gene transfer and the origin of species: lessons from bacteria**. *Trends Microbiol* 2000, **8**:128-133.

5. Salyers AA, Shoemaker NB, Stevens AM, Li LY: **Conjugative transposons — an unusual and diverse set of integrated gene-transfer elements**. *Microbiol Rev* 1995, **59**:579.

6. Sommer MO, Dantas G: **Antibiotics and the resistant microbiome**. *Curr Opin Microbiol* 2011, **14**:556-563.

7. •• Sommer MO, Dantas G, Church GM: **Functional characterization of the antibiotic resistance reservoir in the human microflora**. *Science* 2009, **325**:1128-1131.

The first application of metagenomic functional selections to study the resistome of the human microbiota. The authors identify a diverse set of novel resistance genes, while showing that resistance genes identical to those of human pathogens are harbored by cultivated strains of the human microbiota.

8. Moore AM, Munck C, Sommer MOA, Dantas G: **Functional metagenomic investigations of the human intestinal microbiota**. *Front Microb* 2011, **2**.

9. Vignaroli C, Zandri G, Aquilanti L, Pasquaroli S, Biavasco F: **Multidrug-resistant enterococci in animal meat and faeces and co-transfer of resistance from an *Enterococcus durans* to a human *Enterococcus faecium***. *Curr Microbiol* 2011, **2**:1438-1447.

10. • Martinez JL: **Antibiotics and antibiotic resistance genes in natural environments**. *Science* 2008, **321**:365-367.

An important review of environmental resistomes, highlighting the importance of genetic context in defining resistance.

11. Baquero F, Martinez JL, Canton R: **Antibiotics and antibiotic resistance in water environments**. *Curr Opin Biotechnol* 2008, **19**:260-265.

12. Davies J, Davies D: **Origins and evolution of antibiotic resistance**. *Microbiol Mol Biol Rev* 2010, **74**:417-433.

13. Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J: **Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil**. *ISME J* 2009, **3**:243-251.

14. Walsh C: *Antibiotics: Actions, Origins Resistance*. Washington, DC: ASM Press; 2003.

15. Arthur M, Courvalin P: **Genetics and mechanisms of glycopeptide resistance in enterococci**. *Antimicrob Agents Chemother* 1993, **37**:1563-1571.

16. Wang P, Brobey RK, Horii T, Sims PF, Hyde JE: **Utilization of exogenous folate in the human malaria parasite *Plasmodium falciparum* and its critical role in antifolate drug synergy**. *Mol Microbiol* 1999, **32**:1254-1262.

17. Stewart PS, Costerton JW: **Antibiotic resistance of bacteria in biofilms**. *Lancet* 2001, **358**:135-138.

18. Poirel L, Bonnin RA, Nordmann P: **Analysis of the resistome of a multidrug-resistant NDM-1-producing Escherichia coli strain by high-throughput genome sequencing**. *Antimicrob Agents Chemother* 2011, **55**:4224-4229.

19. Goodman AL, Kallstrom G, Faith JJ, Reyes A, Moore A, Dantas G, Gordon JI: **Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice**. *Proc Natl Acad Sci U S A* 2011, **108**:6252-6257.

20. •• Riesenfeld CS, Goodman RM, Handelsman J: **Uncultured soil bacteria are a reservoir of new antibiotic resistance genes**. *Environ Microbiol* 2004, **6**:981-989.

Seminal paper applying metagenomic functional selections to identify novel resistance genes from the soil microbiota.

21. Jacoby GA: **AmpC beta-lactamases**. *Clin Microbiol Rev* 2009, **22**:161-182 (Table of contents).

22. Perez-Perez FJ, Hanson ND: **Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR**. *J Clin Microbiol* 2002, **40**:2153-2162.

23. Reisbig MD, Hanson ND: **Promoter sequences necessary for high-level expression of the plasmid-associated ampC beta-lactamase gene blaMIR-1**. *Antimicrob Agents Chemother* 2004, **48**:4177-4182.

24. •• Karami N, Martner A, Enne VI, Swerkersson S, Adlerberth I, Wold AE: **Transfer of an ampicillin resistance gene between two *Escherichia coli* strains in the bowel microbiota of an infant treated with antibiotics**. *J Antimicrob Chemother* 2007, **60**:1142-1145.

First in situ demonstration of transfer of antibiotic resistance genes between strains naturally inhabiting the human microbiota of a patient undergoing antibiotic treatment.

25. • D'Costa VM, King CE, Kalan L, Morar M, Sung WW, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R *et al.*: **Antibiotic resistance is ancient**. *Nature* 2011, **477**:457-461.

These authors used metagenomic sequencing to identify a diverse set of genes encoding resistance to β-lactam, tetracycline, and glycopeptide antibiotics from DNA isolated from 30,000-year-old Beringian permafrost sediments, highlighting the ancient origins of antibiotic resistance genes.

26. •• Benveniste R, Davies J: **Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria**. *Proc Natl Acad Sci U S A* 1973, **70**:2276-2280.
This key paper provided the first biochemical support for the 'Producer Hypothesis', showing that soil-dwelling antibiotic-producer Actinomycetes harbor resistance proteins with similar activities to resistance proteins found in pathogens, hence implicating the producers as donors of resistance to pathogens.

27. Dantas G, Sommer MO, Oluwasegun RD, Church GM: **Bacteria subsisting on antibiotics**. *Science* 2008, **320**:100-103.

28. Blodgett JA, Oh DC, Cao S, Currie CR, Kolter R, Clardy J: **Common biosynthetic origins for polycyclic tetramate macrolactams from phylogenetically diverse bacteria**. *Proc Natl Acad Sci U S A* 2010, **107**:11692-11697.

29. • D'Costa VM, McGrann KM, Hughes DW, Wright GD: **Sampling the antibiotic resistome**. *Science* 2006, **311**:374-377.
This paper reports on high-levels of multi-drug resistance in cultured soil isolates of the antibiotic producer *Streptomycete* genus, despite no initial selection for resistance.

30. D'Costa VM, Griffiths E, Wright GD: **Expanding the soil antibiotic resistome: exploring environmental diversity**. *Curr Opin Microbiol* 2007, **10**:481-489.

31. Schnappinger D, Hillen W: **Tetracyclines: antibiotic action, uptake, and resistance mechanisms**. *Arch Microbiol* 1996, **165**:359-369.

32. Thaker M, Spanogiannopoulos P, Wright GD: **The tetracycline resistome**. *Cell Mol Life Sci* 2010, **67**:419-431.

33. Palmer AC, Angelino E, Kishony R: **Chemical decay of an antibiotic inverts selection for resistance**. *Nat Chem Biol* 2010, **6**:244.

34. Rodriguez-Martinez JM, Velasco C, Garcia I, Cano ME, Martinez-Martinez L, Pascual A: **Characterisation of integrons containing the plasmid-mediated quinolone resistance gene qnrA1 in *Klebsiella pneumoniae***. *Int J Antimicrob Agents* 2007, **29**:705-709.

35. Hall RM, Collis CM: **Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination**. *Mol Microbiol* 1995, **15**:593-600.

36. • Kristiansson E, Fick J, Janzon A, Grabic R, Rutgersson C, Weijdegard B, Soderstrom H, Larsson DG: **Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements**. *PLoS One* 2011, **6**:e17038.
This paper highlights the incredibly high selection pressure imposed on the environment due to anthropogenic contamination from inappropriately treated effluents from industrial antibiotic production.

37. Levings RS, Lightfoot D, Partridge SR, Hall RM, Djordjevic SP: **The genomic island SGI1, containing the multiple antibiotic resistance region of Salmonella enterica serovar typhimurium DT104 or variants of it, is widely distributed in other S-enterica serovars**. *J Bacteriol* 2005, **187**:4401-4409.

38. Partridge SR, Recchia GD, Stokes HW, Hall RM: **Family of class 1 integrons related to In4 from Tn1696**. *Antimicrob Agents Chemother* 2001, **45**:3014-3020.

39. Piddock LJ: **Multidrug-resistance efflux pumps — not just for resistance**. *Nat Rev Microbiol* 2006, **4**:629-636.

40. Webber MA, Piddock LJ: **The importance of efflux pumps in bacterial antibiotic resistance**. *J Antimicrob Chemother* 2003, **51**:9-11.

41. Canton R, Coque TM: **The CTX-M beta-lactamase pandemic**. *Curr Opin Microbiol* 2006, **9**:466-475.

42. •• Humeniuk C, Arlet G, Gautier V, Grimont P, Labia R, Philippon A: **Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types**. *Antimicrob Agents Chemother* 2002, **46**:3045-3049.
This paper provides the best current evidence for an environmental progenitor of a plasmid-encoded antibiotic resistance gene which is rapidly disseminating amongst globally distributed pathogens.

43. Poirel L, Kampfer P, Nordmann P: **Chromosome-encoded Ambler class A beta-lactamase of Kluyvera georgiana, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases**. *Antimicrob Agents Chemother* 2002, **46**:4038-4040.

44. Martinez-Martinez L, Pascual A, Jacoby GA: **Quinolone resistance from a transferable plasmid**. *Lancet* 1998, **351**:797-799.

45. • Poirel L, Rodriguez-Martinez JM, Mammeri H, Liard A, Nordmann P: **Origin of plasmid-mediated quinolone resistance determinant QnrA**. *Antimicrob Agents Chemother* 2005, **49**:3523-3525.
This paper reports on one of the very few current examples of an environmental progenitor of plasmid-mediated resistance in pathogens.

46. Poirel L, Liard A, Rodriguez-Martinez JM, Nordmann P: **Vibrionaceae as a possible source of Qnr-like quinolone resistance determinants**. *J Antimicrob Chemother* 2005, **56**:1118-1121.

47. Bonemann G, Stiens M, Puhler A, Schluter A: **Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, qnrS2, isolated from the bacterial community of a wastewater treatment plant**. *Antimicrob Agents Chemother* 2006, **50**:3075-3080.

48. Aminov RI, Mackie RI: **Evolution and ecology of antibiotic resistance genes**. *FEMS Microbiol Lett* 2007, **271**:147-161.

49. Keen PL, Montforts MHMM: *Antimicrobial Resistance in the Environment*. Wiley–Blackwell; 2012.

50. Heuer H, Schmitt H, Smalla K: **Antibiotic resistance gene spread due to manure application on agricultural fields**. *Curr Opin Microbiol* 2011, **14**:236-243.

51. Walsh TR, Weeks J, Livermore DM, Toleman MA: **Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study**. *Lancet Infect Dis* 2011, **11**:355-362.

52. Auerbach EA, Seyfried EE, McMahon KD: **Tetracycline resistance genes in activated sludge wastewater treatment plants**. *Water Res* 2007, **41**:1143-1151.

53. Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, Galland JC, Graham DW: **Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR**. *Appl Environ Microbiol* 2004, **70**:7372-7377.

54. Marshall BM, Levy SB: **Food animals and antimicrobials: impacts on human health**. *Clin Microbiol Rev* 2011, **24**:718-733.

55. McManus PS, Stockwell VO, Sundin GW, Jones AL: **Antibiotic use in plant agriculture**. *Annu Rev Phytopathol* 2002, **40**:443-465.

56. • Knapp CW, Dolfing J, Ehlert PA, Graham DW: **Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940**. *Environ Sci Technol* 2010, **44**:580-587.
This paper demonstrates that levels of specific antibiotic resistance genes in the soil have been steadily increasing over the past 70 years, likely due to anthropogenic activities.

57. Ochman H, Lawrence JG, Groisman EA: **Lateral gene transfer and the nature of bacterial innovation**. *Nature* 2000, **405**:299-304.

58. Elshahed MS, Youssef NH, Spain AM, Sheik C, Najar FZ, Sukharnikov LO, Roe BA, Davis JP, Schloss PD, Bailey VL *et al.*: **Novelty and uniqueness patterns of rare members of the soil biosphere**. *Appl Environ Microbiol* 2008, **74**:5422-5428.

59. Daniel R: **The metagenomics of soil**. *Nat Rev Microbiol* 2005, **3**:470-478.

60. Sommer MO, Church GM, Dantas G: **A functional metagenomic approach for expanding the synthetic biology toolbox for biomass conversion**. *Mol Syst Biol* 2010, **6**:360.

61. •• Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G: **The shared antibiotic resistome of soil bacteria and human pathogens**. *Science* 2012 http://dx.doi.org/10.1126/science.1220761.
This paper demonstrates for the first time that nonpathogenic soil bacteria share numerous resistance genes of diverse mechanisms with globally distributed human pathogens, supporting recent exchange of resistance genes between these reservoirs.